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<b>(54) Title:</b> METHODS OF USING BIOELASTOMERS  <b>(57) Abstract</b>  Bioelastomers are disclosed for use in methods of binding compounds including immunoassay methods, in biosensors and methods of regenerating biosensors, and in methods for targeting the delivery of a compound to a particular location within animal subjects. In general, the bioelastomer is conjugated to a binding compound, which is in turn used to bind a compound of interest. For targeted compound delivery, the bioelastomer is conjugated to the compound to be delivered.		

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## METHODS OF USING BIOELASTOMERS

Field of the Invention

The present invention concerns bioelastic polymers and methods of use thereof, particularly for immunoassays, for the production of regenerable biosensors, and for the localized delivery of compounds *in vivo*.

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Background of the Invention

Numerous bioelastic polymers, also known as bioelastomers or elastin-like proteins ("ELPs") are known. Examples are described in D. Urry et al., *A Simple Method for the Purification of a Bioelastic Polymer*, PCT Application WO 96/32406. Such compounds are proteins or peptides, typically polypeptides, that exhibit an inverse temperature transition: that is, the compounds condense at a higher temperature range in an aqueous system on raising the temperature of the compounds through their transition temperature ( $T_I$ ). Bioelastic polymers are soluble in water at a sufficiently low temperature, but hydrophobically fold and associate to form a separate phase as the temperature is raised through a particular temperature range.

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1. Separation and Immunoassay Systems.

Immunoassays are commonly used to detect analytes such as enzymes, hormones, drugs, and other molecules of interest in complex biological mixtures. By definition, an immunoassay relies on the specific binding of an antigen by an antibody, but assays using other biological high affinity binding partners (e.g., ligand-cell surface receptor, inhibitor-enzyme, etc.) can also be employed in an analogous manner. A number of different immunoassay formats have been developed to detect and/or quantitate the levels of analytes; for a review of immunoassays, see PCT Patent Application WO 86/06492.

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Ligand-binding proteins such as receptors and antibodies, currently used in biosensors, can detect specific analytes (ligands) with high sensitivity in the presence of potential interference's in complex mixtures. The high affinity of protein-analyte interactions is the basis of their exquisite sensitivity. However, high affinity is generally accompanied by an extremely slow dissociation rate (off rate) of the protein-ligand complex. Therefore, in practice, most biosensors are "one shot" devices; dosimeters rather than continuous sensors or alternatively, sensors with very slow response times. In order to use biosensors for the semi-continuous, *in situ* monitoring of analytes, or for subsequent rounds of sensing in batch mode, the sensor must be regenerated for reuse in an expedient time frame.

There are two possible approaches to sensor regeneration. When the receptor is covalently coupled to the sensor surface, free receptor can be regenerated by displacing the bound analyte. Unfortunately, methods to gently and reversibly regenerate analyte-free receptor do not currently exist: most current methods disrupt noncovalent interactions between analyte and receptor by partially denaturing the receptor using drastic changes in the protein-ligand environment such as low pH (<3), or high chaotrope concentration, conditions which often irreversibly denature the protein after a few rounds of regeneration.

If the receptor is not covalently attached to the substrate, a second method for surface regeneration is feasible where the surface itself can be regenerated by removing the analyte-bound receptor from the surface. The potential advantage is that the analyte "sees" fresh receptor in every round of sensing, which can decrease drift in the sensor response and maintain high affinity and homogenous binding kinetics. This approach to sensor regeneration is difficult to broadly implement because noncovalent methods to immobilize proteins on surfaces typically involves their physical adsorption, which is typically irreversible, and subsequent stripping of adsorbed protein with detergents or chaotropes is frequently incomplete. In order to noncovalently and reversibly bind a receptor to the surface, methods must be found to reversibly control the physico-chemical properties of the receptor such that the adsorption-desorption process can be triggered reversibly.

### **3. Targeted Delivery of Therapeutics to Solid Tumors by Thermally-Responsive Polymers.**

The targeted delivery of drugs to solid tumors is a complex problem because of the impediments to drug delivery that are posed by tumor heterogeneity. Cancer cells typically occupy less than half of the total tumor volume. Approximately 1-10% is contributed by tumor vasculature, and the rest is occupied by a collagen-rich interstitium. The major impediments to drug delivery arise from heterogeneous distribution of blood vessels, combined with aberrant branching and tortuosity, which results in uneven and slowed blood flow. The leakiness of tumor vessels combined with the absence of a functional lymphatic system results in an elevated interstitial pressure, which retards the convective transport of high MW (>2000 Da) drugs. R. Jain, *Sci. Am.* **271**: 58-65 (1994). The heterogeneity of antigen and receptor expression in tumors is an additional problem in affinity-targeted delivery of drugs to solid tumors.

Front-line therapies for different tumors include surgery, chemotherapy, and radiation. The infiltrative nature of many solid tumors often prevents complete surgical resection because of the high risk of compromising function, thereby necessitating postoperative chemotherapy and/or radiotherapy. However, chemotherapy, particularly when delivered systemically is of limited effectiveness due to inadequate drug delivery, systemic toxicity, and a markedly variable biological sensitivity. External beam irradiation, while useful for many types of tumors, is also limited by dose limiting toxicity to healthy tissue.

Two other treatment modalities that have been suggested for the treatment of solid tumors, are hyperthermia [S. Field and J. Hand, *An Introduction to the Practical Aspects of Clinical Hyperthermia* (Taylor and Francis, London 1990)] and targeted radiotherapy [C. Hoefnagel, *Int. J. Biol. Markers* **8**: 172 (1993); M. Gaze, *Phys. Med. Biol.* **41**: 1895 (1993)]. The use of local hyperthermia as a therapeutic modality for solid tumors is motivated by the increased thermal sensitivity of tumor vasculature compared to normal vasculature. Hyperthermia, at temperatures between 40 and 42 °C, is known to increase tumor blood flow and vascular permeability. Because

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to increase tumor cytotoxicity [M. Hauck et al., *in* Handbook of Targeted Delivery of Imaging Agents, pp. 335-361 (V. Torchilin Ed. 1995)].

The limitations of current therapeutic approaches for the management of solid tumors provide a compelling need for the development of improved modalities for the  
5 targeted delivery of therapeutics.

### Summary of the Invention

A first aspect of the present invention is a method of binding a compound of interest in an aqueous solution. The method comprises the steps of:

10 (a) providing a conjugate comprising a bioelastic compound and a binding compound, wherein the binding compound specifically binds the compound of interest, and wherein the bioelastic compound has a transition temperature below which the bioelastic compound is soluble in the solution, and above which the bioelastic compound is insoluble in the solution;

15 (b) contacting the conjugate to the compound of interest in an aqueous solution so that the compound binds thereto, with the contacting step carried out at a temperature below the transition temperature of the bioelastic compound; and then

(c) raising the temperature of the conjugate to a temperature above the transition temperature of the bioelastic compound so that the conjugate separates from  
20 the aqueous solution with the compound of interest bound thereto. Separation may be passive (*e.g.*, precipitation in solution) or active (*e.g.*, by centrifugation or filtration). Thus, the step of "raising the temperature" may be thought of as being followed by a step of separating (*i.e.*, actively separating) the conjugate with the compound of interest bound thereto from the solution.

25 A second aspect of the present invention is a method useful for immunologically detecting an analyte in an aqueous solution. The method comprises the steps of:

(a) providing a conjugate comprising a bioelastic compound and a binding compound, wherein the binding compound specifically binds the analyte, and wherein  
30 the bioelastic compound has a transition temperature below which the bioelastic compound is soluble in the solution, and above which the bioelastic compound is

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(b) contacting the conjugate to the analyte in an aqueous solution so that the compound binds thereto, with the contacting step carried out at a temperature below the transition temperature of the bioelastic compound;

(c) raising the temperature of the conjugate to a temperature above the transition temperature of the bioelastic compound so that the conjugate separates from the aqueous solution with the analyte bound thereto; and then

(d) detecting the analyte.

A third aspect of the present invention is an article useful as a regenerable biosensor for binding a compound of interest from an aqueous solution, or for any such other purposes to which the article may be suitable. The article comprises:

(a) a solid support having a hydrophobic surface formed thereon; and

(b) a conjugate reversibly bonded to the hydrophobic surface, the conjugate comprising (i) a bioelastic compound and a (ii) binding compound, wherein the binding compound specifically binds the compound of interest, and wherein the bioelastic compound has a transition temperature below which the bioelastic compound is soluble in the solution, and above which the bioelastic compound is insoluble in the solution; so that the biosensor may be used to bind the compound of interest at a temperature above the transition temperature, and so that the conjugate can be removed from the solid support for recycling of the article by lowering the temperature of the solid support (and/or the solution; so long as the temperature of the conjugate is lowered) below the transition temperature.

A fourth aspect of the invention is a method of recycling a used biosensor to which has been bound a compound of interest. The method comprises:

(a) providing a biosensor comprising a solid support having a hydrophobic surface formed thereon and a first conjugate reversibly bonded to the hydrophobic surface, the conjugate comprising (i) a bioelastic compound (which is bonded to the hydrophobic surface by hydrophobic interactions when at a temperature above its transition temperature) and (ii) a binding compound, wherein the binding compound has the compound of interest specifically bound thereto, and wherein the bioelastic compound has a transition temperature below which the bioelastic compound is soluble in the solution, and above which the bioelastic compound is insoluble in the

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(b) separating the conjugate with the compound of interest bound thereto from the solid support by lowering the temperature of the biosensor to below the transition temperature; and then

(c) binding a second conjugate to the hydrophobic surface, the second  
5 conjugate comprising a second bioelastic compound and a second binding compound, so that the biosensor may be reused. The second conjugate may be the same as or different from the first conjugate.

A fifth aspect of the present invention is a method for the targeted delivering of a compound *in vivo* to a selected region within a subject. The method comprises:

10 (a) administering a conjugate to the subject, the conjugate comprising the compound to be delivered and a polymer that undergoes an inverse temperature transition, wherein the polymer has a transition temperature ( $T_i$ ) greater than the temperature at which the compound is delivered; and then

(b) heating the selected region to a temperature greater than the transition  
15 temperature of the polymer, so that the compound is preferentially delivered to the selected region.

A sixth aspect of the present invention is the use of a conjugate as described above for the preparation of a medicament for the targeted delivery of a compound as described above.

20 The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification below.

### **Brief Description of the Drawings**

**Figure 1** illustrates the Expression construct in pET-32b, which includes two  
25 N-terminal domains: thioredoxin, and a (His)<sub>6</sub> tag.

**Figure 2a** illustrates an immunoassay with no thioredoxin-ELP (and hence no specific antibody capture);

**Figure 2b** illustrates an immunoassay with 10:1 thioredoxin-ELP:antibody ratio (and hence nearly complete antibody capture);

30 **Figure 3** illustrates an immunoassay calibration curve. The X-axis is the ratio of unlabeled to total antibody concentrations, with points corresponding to unlabeled



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the ratio of fluorescence in the pellet to total fluorescence (pellet + supernatant), and represents the fraction of labeled anti-thioredoxin antibodies specifically captured.

**Figure 4.** Absorbance at 350 nm of the thioredoxin-ELP 18-mer by UV-visible spectrophotometry in 50 mM phosphate, pH 7.4, 1 M NaCl. Relative turbidity is defined as the absorbance at 350 nm (due to scattering) normalized to the maximum absorbance at the same wavelength during the course of the experiment.

**Figure 5.** *In situ* ellipsometry measurements of the change in adlayer thickness of the thioredoxin-ELP fusion protein in contact with a hydrophobic SAM as a function of temperature, calculated by nonlinear regression using the measured values of the polarizer and analyzer angles. The thickness of the protein adlayer measured by ellipsometry increases as the temperature is raised through the  $T_1$  to a maximum of 180 Å, and decreases to within 5 Å of the baseline thickness upon lowering the temperature to ~18 °C.

**Figure 6** illustrates relative SPR signal as a function of recording time of the experiment. The recording time is not identical to the experimental time, since some time segments such as flushing, buffer exchange, etc. are not included, to enhance clarity.

**Figure 7** illustrates desorption of thioredoxin-ELP fusion protein monitored by the SPR signal relative to control channel as a function of temperature.

**Figure 8.** Absorbance at 350 nm of the ELP 6-mer (filled squares), 9-mer (open squares), and 18-mer (filled triangles) as a function of temperature.

**Figure 9.** Absorbance at 350 nm of the ELP 15-mer (filled squares) and poly(NIPAAm/AAm) copolymer (open squares) by UV-visible spectrophotometry. Assay conditions: 1 mg/ml, PBS, pH 7.2 heating rate = ~1.0 °C/min.

**Figure 10.** Absorbance at 350 nm of the ELP 15-mer (filled squares) and Rhodamine-ELP 15 mer conjugate (filled circles) by UV-visible spectrophotometry. Assay conditions: 1 mg/ml, PBS, pH 7.2 heating rate = ~1.0 °C/min.

**Figure 11A.** Fluorescence intensities of Rhodamine-ELP conjugate in tumor window chamber as a function of time post injection under hyperthermic conditions (42 °C). The fluorescence intensity of the control vascular region (I<sub>c</sub>) (open squares) and

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interstitial region ( $I_i$ ) (filled squares) are shown normalized to the maximum vascular concentration.

**Figure 11B.** Fluorescence intensities of Rhodamine-ELP conjugate in tumor window chamber as a function of time post injection under normothermic conditions (34 °C). The light intensity of a representative vascular region ( $I_v$ ) (open squares) and interstitial region ( $I_i$ ) (filled squares) are shown normalized to the maximum vascular concentration.

**Figure 12A** shows a fluorescence image of a tumor under hyperthermic conditions (42 °C).

**Figure 12B** shows a fluorescence image of a tumor under normothermic control (34 °C).

#### Detailed Description of Preferred Embodiments

“Compound of interest” as used herein refers to any compound, including proteins, peptides, nucleic acids particularly polynucleic acids (*e.g.*, DNA, RNA), organic compounds, etc. Such compounds may be antibodies, receptors, ligands, hormones, drugs, etc. When the method of the invention is employed to qualitatively or quantitatively detect the compound of interest, the compound of interest may be referred to as an “analyte”. The compound of interest or analyte may be carried by a cell (particularly on the surface of a cell) so that a cell (or cell of interest) is bound to the binding partner through the compound of interest.

“Binding compound” as used herein refers to a compound that specifically or selectively binds the compound of interest. In general, the binding compound and the compound of interest together comprise a specific binding pair. The binding compound may be any compound, including proteins, peptides, nucleic acids particularly polynucleic acids (*e.g.*, DNA, RNA), organic compounds, etc. Such compounds may be antibodies, receptors, ligands, hormones, drugs, etc.

“Conjugate” as used herein refers to two moieties or functional groups that are covalently or noncovalently joined to one another, such that the two groups function together as a single structure under the conditions of the methods described herein. In one embodiment, the conjugate is a fusion protein. The binding compound may be

undergoes an inverse temperature transition through an antibody or other binding partner with the cell.

"Fusion protein" as used herein refers to a protein or peptide, produced by recombinant means (*i.e.*, expression from a nucleic acid) that is comprised of a first protein or peptide covalently joined on expression to a second protein or peptide.

A "polymer that undergoes an inverse temperature transition" herein refers to a polymer that is soluble in an aqueous solution at a lower temperature, and is insoluble in an aqueous solution at a higher temperature.

"Transition temperature" or " $T_1$ " as used herein, refers to the temperature above which a polymer that undergoes an inverse temperature transition is insoluble in an aqueous system (*e.g.*, water, physiological saline solution), and below which such a polymer is soluble in an aqueous system. Preferred  $T_1$ s for *in vitro* applications of the invention are 0° or 20° to 60° or 100°C; preferred  $T_1$ s for *in vivo* applications are 35° to 45° C.

A "bioelastic polymer" is, in general, a polypeptide that exhibits an inverse temperature transition. Bioelastic polymers are discussed in greater detail below.

"Poly(N-isopropylacrylamide) polymer" as used herein refers to a class of polymers, including copolymers thereof, that exhibit an inverse temperature transition. The polymers are formed of at least N-isopropylacrylamide, and may include other monomers that polymerize therewith, such as acrylamide (Aam), methacrylamide, propylacrylamide, butylacrylamide, acrylate, methacrylate, etc.

### 1. Bioelastic polymers.

Bioelastic polymers are, in general, polypeptides that comprise repeating units that form beta-turns. A beta-turn is a 10 atom hydrogen-bonded ring in which the C=O of amino acid residue *i* is hydrogen-bonded to the NH residue of amino acid *i*+3. Repetitive beta-turns result in elastic beta-spiral structures. Bioelastic polymers, in general, are soluble in water at a sufficiently low temperature, but hydrophobically fold and associate to form a separate phase as the temperature is raised through the transition temperature. When more hydrophobic amino acids are included in the bioelastic polymer the transition temperature is lower; when fewer hydrophobic

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temperature. The chain length of the bioelastic polymer is not critical, but typically is from 10 or 20 to 50, 100, 200, or 1000 or more amino acids. Numerous bioelastic polymers are known and described in, for example, U.S. Patents Nos. 4,132,746; 4,187,852; 4,589,822; 4,870,055; and 5,064,430 (applicants specifically intend that the disclosures of all patent references cited herein be incorporated herein by reference in their entirety). A particularly preferred bioelastic polymer is one that contains the elastin repeat: (Val-Pro-Gly-X-Gly)<sub>n</sub> or (VPGXG)<sub>n</sub>, where n is from 2, 3 or 4 to 10, 20, 30 40, 100 or 200 or more, and X is any amino acid, such as valine, alanine or glycine, with X being the same or different in each repeat unit of the polypeptide chain (that is, X may be a combination of different amino acids, in various ratios).

## 2. Separation and Immunoassay Systems.

As noted above, the present invention provides a method of binding a compound of interest in an aqueous solution, the method comprising the steps of:

(a) providing a conjugate comprising a bioelastic compound and a binding compound, wherein the binding compound specifically binds the compound of interest, and wherein the bioelastic compound has a transition temperature below which the bioelastic compound is soluble in the solution, and above which the bioelastic compound is insoluble in the solution;

(b) contacting the conjugate to the compound of interest in an aqueous solution so that the compound binds thereto, with the contacting step carried out at a temperature below the transition temperature of the bioelastic compound; and then

(c) raising the temperature of the conjugate to a temperature above the transition temperature of the bioelastic compound so that the conjugate separates from the aqueous solution with the compound of interest bound thereto.

As noted above, the binding compound may be any suitable molecule, such as a protein or peptide. The binding compound may be an antibody. Likewise, the compound of interest, may be a protein or peptide such as an antibody.

When the method is used as an immunoassay, the compound of interest is referred to as an analyte, and the binding step is followed by the step of detecting the

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critical and will depend upon the particular immunoassay format. The present invention can be implemented with any immunoassay format, including direct immunoassays, indirect immunoassays, and sandwich assays; and heterogeneous or homogeneous immunoassays, numerous variations of which are known to persons skilled in the art.

### **3. Biosensor Systems and Biosensor Regeneration.**

As also noted above, the present invention provides an article useful as a regenerable biosensor for binding a compound of interest from an aqueous solution, the article comprising:

(a) a solid support having a hydrophobic surface formed thereon; and  
(b) a conjugate reversibly bonded to the hydrophobic surface, the conjugate comprising (i) a bioelastic compound and a (ii) binding compound, wherein the binding compound specifically binds the compound of interest, and wherein the bioelastic compound has a transition temperature below which the bioelastic compound is soluble in the solution, and above which the bioelastic compound is insoluble in the solution; so that the biosensor may be used to bind the compound of interest at a temperature above the transition temperature, and so that the conjugate can be removed from the solid support for recycling of the article by lowering the temperature of the solid support (or the solution in which the solid support is carried, so long as the temperature of the bioelastic compound is lowered) below the transition temperature. Again, the binding compound may be a protein or peptide, such as an antibody, and the compound of interest may be a protein or peptide, such as an antibody.

The present invention can be utilized with any type of biosensor where it is desirable to regenerate a solid support that has a binding compound immobilized thereon. For example, the present invention may be utilized with optical biosensors such as described in U.S. Patents Nos. 5,313,264 to Ulf et al., 5,846,842 to Herron et al., 5,496,701 to Pollard-Knight et al., etc. The present invention may be utilized with potentiometric or electrochemical biosensors, such as described in U.S. Patent No. 5,413,690 to Kost, or PCT Application WO98/35232 to Fowlkes and Thorp. The

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inorganic; may be metal (*e.g.*, copper or silver) or non-metal; may be a polymer or nonpolymer; may be conducting, semiconducting or nonconducting (insulating); may be reflecting or nonreflecting; may be porous or nonporous; etc. For example, the solid support may be comprised of polyethylene, polytetrafluoroethylene, gold, silicon, silicon oxide, silicon oxynitride, indium, platinum, iridium, indium tin oxide, diamond or diamond-like film, etc. When the solid support does not inherently provide a hydrophobic surface to which the bioelastic polymer may reversibly bind (*i.e.*, non-covalently, primarily by means of hydrophobic interactions), the surface may be functionalized or coated to render it hydrophobic in accordance with any of a variety of standard techniques.

The present invention may be utilized with different binding compounds or compounds of interest desired to be bound to a substrate, for the high throughput screening of molecular interactions, such as in "chip-based" and "pin-based" combinatorial chemistry techniques, and can be prepared by modification of known techniques based on the disclosure provided herein. In such case the substrate or solid support has a surface portion, with the surface portion comprising a plurality of discrete known regions (each of which has a hydrophobic surface portion formed thereon). A plurality of different conjugates (differing in the binding compound, and optionally in the bioelastic compound thereof) are bound to the surface portion, with different conjugates positioned on the surface portion in different ones of the discrete known regions. All can be prepared in accordance with known techniques. *See, e.g.*, U.S. Patent No. 5,445,934 to Fodor et al., U.S. Patent No. 5,288,514 to Ellman, and U.S. Patent No. 5,624,711 to Sundberg et al., the disclosures of which are incorporated by reference herein in their entirety.

Thus the present invention provides a method of recycling a biosensor as described above by:

(a) providing a biosensor comprising a solid support having a hydrophobic surface formed thereon and a first conjugate reversibly bonded to the hydrophobic surface, the conjugate comprising (i) a bioelastic compound and (ii) a binding compound, wherein the binding compound has the compound of interest specifically bound thereto, and wherein the bioelastic compound has a transition temperature

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(b) separating the conjugate with the compound of interest bound thereto from the solid support by lowering the temperature of the biosensor (or the solution in which the sensor is carried, so long as the temperature of the bioelastic compound is lowered) to below the transition temperature; and then

- 5       (c) binding a second conjugate to the hydrophobic surface, the second conjugate comprising a second bioelastic compound and a second binding compound, so that the biosensor may be reused. Again, the second conjugate may be the same or different from the first conjugate, and either or both of the second bioelastic compound and second binding compound may be different from the first.

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#### 4. Targeted Delivery of Therapeutics to Solid Tumors by Thermally-Responsive Polymers.

- As discussed above, a method for the targeted delivering of a compound *in vivo* to a selected region within a subject comprises: (a) administering a conjugate to  
15   the subject, the conjugate comprising the compound to be delivered and a polymer that undergoes an inverse temperature transition, wherein the polymer has a transition temperature ( $T_i$ ) greater than the temperature at which the compound is delivered; and then (b) heating the selected region to a temperature greater than the transition temperature of the polymer, so that the compound is preferentially delivered to the  
20   selected region. The polymer may be a bioelastic polymer as described above, or may be a poly(N-isopropylacrylamide) polymer as described above.

- While the present invention is concerned primarily with the treatment of human subjects, the invention may also be used for the treatment of animal subjects, particularly mammalian subjects such as dogs, cats, horses, cows, pigs, etc., for  
25   veterinary purposes.

- Administering of the conjugate to the subject may be carried out by any suitable means, such as subcutaneous injection, intraperitoneal injection, intravenous injection, intramuscular injection, oral administration, inhalation administration, transdermal administration, etc. Preferred administration techniques are typically  
30   "systemic" in that a particular region of interest is not specifically targeted.

The selected region may be any suitable target or portion of the subject's

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such as a solid tumor). Examples of tumors, cancers and neoplastic tissue that can be treated by the present invention include but are not limited to malignant disorders such as breast cancers; osteosarcomas; angiosarcomas; fibrosarcomas and other sarcomas; leukemias; lymphomas; sinus tumors; ovarian, ureteral, bladder, prostate and  
5 other genitourinary cancers; colon esophageal and stomach cancers and other gastrointestinal cancers; lung cancers; myelomas; pancreatic cancers; liver cancers; kidney cancers; endocrine cancers; skin cancers; and brain or central and peripheral nervous (CNS) system tumors, malignant or benign, including gliomas and neuroblastomas. Examples of premalignant and nonneoplastic hyperproliferative  
10 disorders include but are not limited to myelodysplastic disorders; cervical carcinoma-in-situ; familial intestinal polyposis such as Gardner syndrome; oral leukoplakias; histiocytoses; keloids; hemangiomas; etc.

Heating of the selected region may be carried out by any means, such as by application of an heat source *e.g.*, a heat pad, a hot water bath, infrared heating lamps,  
15 etc., or by heating means such as directing microwave, ultrasound or other radio frequency energy at the selected region, etc.

Any suitable compound for which targeted delivery is desired may be administered by this means, including imaging agents (or contrast agents) and therapeutic agents.

20 In a preferred embodiment, the therapeutic agent is a radionuclide. Any radionuclide, whether it be for therapeutic or imaging purposes, may be employed, including but not limited to,  $^{131}\text{I}$ ,  $^{90}\text{Y}$ ,  $^{211}\text{At}$ ,  $^{212}\text{Bi}$ ,  $^{67}\text{Cu}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ , and  $^{212}\text{Pb}$ .

The therapeutic agent may be a chemotherapeutic agent or cytotoxic agent. Examples of chemotherapeutic agents which may be coupled to the conjugate include,  
25 but are not limited to, methotrexate, adriamycin, doxorubicin, taxol, etc.. Examples of cytotoxic agents which may be coupled to the conjugate include, but are not limited to, ricin, (or more particularly the ricin A chain).

Imaging agents may be fluorescent compounds such as rhodamine or green fluorescent protein (GFP) or a radionuclide such as  $^{111}\text{In}$ .

30 Coupling of conjugates may be carried out by any suitable means, such as by recombinant means where elastin is joined to a protein or peptide such as GFP; by



The conjugates (or "active compounds") described above may be formulated for administration in a single pharmaceutical carrier or in separate pharmaceutical carriers for the treatment of a variety of conditions. In the manufacture of a pharmaceutical formulation according to the invention, the active compounds  
5 including the physiologically acceptable salts thereof, or the acid derivatives of either thereof are typically admixed with, *inter alia*, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid, or both, and is preferably formulated with the compound as a unit-dose  
10 formulation, for example, a tablet, which may contain from 0.5% to 95% by weight of the active compound. One or more active compounds may be incorporated in the formulations of the invention, which may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory ingredients.

15 The formulations of the invention include those suitable for oral, rectal, topical, buccal (e.g., sub-lingual), parenteral (e.g., subcutaneous, intramuscular, intradermal, or intravenous) and transdermal administration, although the most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular active compound which is  
20 being used.

Formulations suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges, or tablets, each containing a predetermined amount of the active compound; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil  
25 emulsion. Such formulations may be prepared by any suitable method of pharmacy which includes the step of bringing into association the active compound and a suitable carrier (which may contain one or more accessory ingredients as noted above). In general, the formulations of the invention are prepared by uniformly and intimately admixing the active compound with a liquid or finely divided solid carrier,  
30 or both, and then, if necessary, shaping the resulting mixture. For example, a tablet may be prepared by compressing or molding a powder or granules containing the

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free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s). Molded tablets may be made by molding, in a suitable machine, the powdered compound moistened with an inert liquid binder.

- 5           Formulations of the present invention suitable for parenteral administration conveniently comprise sterile aqueous preparations of the active compound, which preparations are preferably isotonic with the blood of the intended recipient. These preparations may be administered by means of subcutaneous, intravenous, intramuscular, or intradermal injection. Such preparations may conveniently be  
10 prepared by admixing the compound with water or a glycine buffer and rendering the resulting solution sterile and isotonic with the blood.

Formulations suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal  
15 administration may also be delivered by iontophoresis (*see*, for example, *Pharmaceutical Research* 3 (6):318 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound. Suitable formulations comprise citrate or bis/tris buffer (pH 6) or ethanol/water and contain from 0.1 to 0.2M active ingredient.

- 20           The therapeutically effective dosage of any one active agent, the use of which is in the scope of present invention, will vary somewhat from compound to compound, patient to patient, and will depend upon factors such as the condition of the patient and the route of delivery. Such dosages can be determined in accordance with routine pharmacological procedures known to those skilled in the art, particularly  
25 in light of the disclosure provided herein. In one example, the dosage is from 1 to 10 micrograms of active compound per Kilogram subject body weight.

In another example, where the therapeutic agent is  $^{131}\text{I}$ , the dosage to the patient is typically from 10 mCi to 100, 300 or even 500 mCi. Stated otherwise, where the therapeutic agent is  $^{131}\text{I}$ , the dosage to the patient is typically from 5,000  
30 Rads to 100,000 Rads (preferably at least 13,000 Rads, or even at least 50,000 Rads). Doses for other radionuclides are typically selected so that the tumoricidal dose is

The present invention is explained in greater detail in the following non-limiting Examples

5

## EXAMPLE 1

### Production of a Thioredoxin-ELP Fusion Protein

#### And Poly-NIPAAm/Am Copolymer

**Gene Synthesis.** The synthetic gene for the ELP-1 mer was constructed from four 5'-phosphorylated, PAGE purified synthetic oligonucleotides (Integrated DNA Technologies, Inc.), ranging in size from 86 to 97 bases. The oligonucleotides were annealed (100 pmol of each oligonucleotide in 50  $\mu$ l ligase buffer, NEB Inc.) to form double-stranded DNA spanning the ELP gene with EcoRI and HindIII compatible ends. The annealed oligonucleotides (2 pmol) were then ligated using T4 DNA ligase (10:1 insert : vector molar ratio) into EcoRI-HindIII linearized and dephosphorylated pUC-19 (NEB, Inc.). Chemically competent *E. Coli* XL1-Blue cells were then transformed with the ligation mixture, and incubated on ampicillin-containing agar plates. Colonies were initially screened by blue-white screening, and subsequently by colony PCR to verify the presence of an insert. The DNA sequence of a putative insert was verified by automated fluorescent DNA sequencing (ABI 373 DNA Sequencer).

**Gene Oligomerization.** First, a 2-mer was created by ligating a 1-mer into a vector containing a 1-mer insert. This process was repeated to create a library of mers ranging from 2 to 9-mer. For a typical oligomerization, the vector was linearized with PflMI, and enzymatically dephosphorylated. The insert was doubly digested with PflMI and BglII, purified by agarose gel electrophoresis (Qiaex II Gel Extraction Kit, Qiagen Inc.), ligated into the linearized vector with T4 DNA ligase at a 5:1 insert : vector molar ratio, and transformed into chemically competent *E. coli* XL1-Blue cells. Transformants were screened by colony PCR, and oligomerization of the 2-mer and 3-mer was further confirmed by DNA sequencing. Standard molecular biology protocols were used for gene synthesis and oligomerization (F. Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley & Sons 1995)).

**Protein Expression.** The genes were subcloned from pUC-19 into a modified expression vector derived from pET-32b (Novagen Inc.). After confirmation of successful cloning by colony PCR and DNA sequencing, pET-32b containing the ELP insert were transformed into the expression strain, BLR21(DE3) (Novagen, Inc.).

5 2xYT media, supplemented with 100 µg/ml ampicillin, were inoculated with transformed cells, incubated at 37° C with shaking (250 rpm), and induced at an OD<sub>600</sub> of 0.6 by the addition of isopropyl α-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture were incubated an additional 3 hours, harvested

10 4° C. Soluble and insoluble fractions of the cell lysate were then characterized by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (U. Lamml, *Nature* 227: 680 (1971)).

**ELP Expression and Purification.** The ELP of interest was expressed as a C-terminal fusion, with thioredoxin and a (His)<sub>6</sub> tag at the N-terminus with an

15 intervening thrombin cleavage site (**Figure 1**). The fusion protein was initially purified by immobilized metal ion affinity chromatography (IMAC) using a Nickel-chelating nitrilotriacetic derivatized resin (Novagen Inc.) (J. Porath, *Prot. Expr. Purif.* 3: 262 (1992)). Alternatively, the fusion protein was purified by repeated cycles of thermal- or salt-induced aggregation, followed by centrifugation, and resolubilization

20 under conditions of low salt or temperature such that the solution temperature was below the inverse transition of the ELP fusion protein. After purification, the soluble ELP-thioredoxin fusion was cleaved by incubation with thrombin, and the ELP was purified by salt-or temperature cycling.

**Synthesis and Characterization of Poly(NIPAAm) copolymer.** The T<sub>i</sub> of

25 poly(NIPAAm) can be adjusted by copolymerizing NIPAAm with acrylamide homologs. Based on previous studies which have reported on this effect (C. Chiklis and J. Grasshof, *J. Polym. Sci. Part A-2* 8: 1617 (1970); L. Taylor and L. Cerankowski, *J. Polym. Sci. Polym. Chem.* 13: 2551 (1975)), we synthesized ~3 g. of an amine-terminated poly(NIPAAm/AAm) copolymer containing 16.6 mol% AAm

30 by free radical copolymerization of NIPAAm and AAm in methanol at 60 °C for 20 h using 1 mmol % of 2,2'-azobisisobutyronitrile (AIBN) as initiator and 1 mmol % of 2-

*Bioconj. Chem.* **4**: 42 (1993). The copolymer was purified by precipitating the reaction solution into diethyl ether and dried under vacuum. The molecular weight distribution of this copolymer was determined by gel permeation chromatography using monodisperse poly(ethylene glycol) standards to calibrate the MW. The polymer has a weight average MW of 20,800 Da and a polydispersity of 2.28.

**Inverse Transition Temperature.** The optical absorbance at 500 nm of the ELP biopolymer or poly(NIPAAm/AAm) copolymer was monitored in the 4-80° C range on a Shimadzu-2001 UV-visible spectrophotometer, equipped with a water-jacketed optical cell connected to a recirculating water bath. The  $T_I$  was determined from the midpoint of the change in optical absorbance at 350 nm due to aggregation of ELP or ELP-thioredoxin fusion protein as a function of temperature at a heating or cooling rate of 1.3 °C min<sup>-1</sup>.

## EXAMPLE 2

### Immunoassay with a Thioredoxin-ELP Fusion Protein

Immunoassays are commonly used to detect analytes such as enzymes, hormones, drugs, and other molecules of interest in complex biological mixtures. By definition, an immunoassay relies on the specific binding of an antigen by an antibody, but assays using other biological high affinity binding partners (e.g., ligand-cell surface receptor, inhibitor-enzyme, etc.) can also be employed in an analogous manner. A number of different immunoassay formats have been developed to detect and/or quantitate the levels of analytes; for a review of immunoassays, see PCT Patent Application WO 86/06492.

Here we report an immunoassay in which one of the reactants (e.g., either the antigen or the antibody) is genetically fused to an elastin-like polypeptide (ELP), which we define as oligomeric polypeptides composed of the pentapeptide repeat Val-Pro-Gly-X-Gly (where X, the guest residue, can be any amino acid). ELPs undergo a reversible phase transition. Below the transition temperature ( $T_I$ ), the polypeptide is highly soluble in aqueous solution; But when the solution temperature is raised above the  $T_I$ , the transition occurs, resulting in aggregation of the polypeptide and partitioning from solution to form a coacervate phase. The aggregated polypeptide

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ultrafiltration. The precipitation is thermally reversible, and upon subsequently resuspending the precipitated polypeptide in an aqueous solution at a temperature below the  $T_i$ , the precipitate completely redissolves. The transition is also sharp, taking place over a 1-2° C range, and can be designed to occur at any temperature  
5 between 0 and 100° C by precisely specifying the guest residue sequence and chain length of the ELP polypeptide, variables that can be exquisitely controlled at the gene level.

In the assay reported here, the reactants were thioredoxin, a soluble *E. coli* protein, and an anti-thioredoxin mouse antibody. A competitive binding assay was  
10 developed in which known amounts of fluorophore-labeled antibody and thioredoxin-ELP fusion protein were incubated with unknown amounts of the analyte (unlabeled antibody), followed by thermally-induced capture of the complex and spectrofluorimetric analysis.

One strength of this immunoassay method is that the ELP is fused to the reactant  
15 genetically rather than by chemical conjugation. This is achieved by joining a gene encoding the polypeptide sequence to a gene of the reactant protein using the techniques of molecular biology. The reactant could be a protein antigen, an antibody or antibody fragment, an enzyme inhibitor, a receptor, or any other protein in an affinity pair of interest. Once constructed, the fused gene can be used in protein  
20 expression systems to produce the ELP-fused immunoassay reactant.

#### A. MATERIALS AND METHODS

**Synthesis of the ELP Gene.** A gene encoding a 50 amino acid sequence was constructed from chemically-synthesized oligonucleotides (Integrated DNA Technologies, Inc.) using standard molecular biology protocols. The 50 amino acid  
25 sequence contained 10 repeats of the pentapeptide VPGXG, where the guest residues (V, A, and G in a 5:2:3 molar ratio) were selected to provide in a  $T_i$  of 40C. The gene was oligomerized end-to-end 15 times by standard molecular biology techniques, to produce an oligomeric ELP gene encoding a 750 residue polypeptide.

**Production of Thioredoxin/ELP Fusion.** The DNA sequence of pET-32b  
30 (Novagen, Inc.), an expression plasmid containing a gene for thioredoxin, was modified to include a *Sfi* I restriction site, which permitted insertion of the ELP gene

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sequence for a thioredoxin-ELP fusion, was transformed into the E. coli strain BLR(DE3) (Novagen Inc.). Shaker flask cultures of the transformed cells were incubated at 37° C to mid-log phase ( $A_{600} \approx 0.800$ ). Protein expression was then induced with 1 mM IPTG, and the cultures were incubated for a further 3 hours. Cells  
5 were lysed by ultrasonication, and the soluble fusion protein was purified from cell lysate by thermally-induced aggregation of the thioredoxin-ELP fusion protein, followed by centrifugation. The fusion protein was subsequently resolubilized in cold buffer at a temperature below the  $T_f$ . Protein purity was ascertained by SDS-PAGE, and protein concentration was determined spectrophotometrically (UV-1601,  
10 Shimadzu Corp.).

**Anti-Thioredoxin Antibody Preparation.** A mouse IgG monoclonal anti-thioredoxin antibody (gift of David Huston, Baylor College of Medicine) was conjugated to fluorescein-5-isothiocyanate (FITC) (Molecular Probes, Inc.). Labeled antibody was then separated from unreacted FITC by gel filtration on a Sephadex G-  
15 25 column (Pharmacia, Inc.). The concentration of antibody was determined spectrophotometrically and by BCA total protein assay (Pierce Chemical Company). The fluorophore to protein ratio in the FITC-labeled antibody was approximately 3.0.

**Immunoassay.** FITC-labeled antibody and thioredoxin-ELP were each added to a concentration of 0.5  $\mu$ M in 50  $\mu$ l PBS. Free ELP (without fused thioredoxin) was  
20 also added to a final concentration of 15  $\mu$ M to facilitate precipitation of the antibody-thioredoxin/ELP complex. Unlabeled antibody (the analyte) was added to the assay mixture in varying amounts, with final concentrations ranging from 5 nM to 5  $\mu$ M.

The analyte-antibody mixtures were incubated at 37° C for 1 hour, then heated to 48° C for five minutes to induce the ELP phase transition, and then centrifuged warm  
25 ( $T > 45^\circ$  C) for 10 minutes at 16,000 g. The supernatant was removed, and the pellet was resuspended in cold PBS. The fluorescence ( $\text{excitation}_{\text{max}} = 494$  nm,  $\text{emission}_{\text{max}} = 519$  nm) of both the supernatant and the resolubilized precipitate were determined by spectrofluorimetry (SLM-Aminco Inc.).

## B. RESULTS AND DISCUSSION

30 Capture of the FITC-labeled antibody by the thioredoxin-ELP fusion was demonstrated in two control experiments, one with no specific capture (Figure 2a)

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the characterization of baseline fluorescence for both the supernatant and pellet fractions.

In **Figure 2a**, the reaction was set up as described in Methods, except that both the unlabeled antibody and thioredoxin-ELP were omitted from the reaction mixture. Because no thioredoxin-ELP was present to capture the FITC-labeled antibody, the precipitate formed after raising the temperature above the  $T_i$  contained only free ELP. Therefore, the majority of the fluorescence remained in the supernatant, with only 3.4% of the total fluorescence observed in the pellet. This small amount of residual fluorescence, which represents the background fluorescence for the pellet fraction of the immunoassay, is likely due to incomplete removal of the supernatant and to non-specific trapping of the labeled antibody in the aggregated ELP phase.

In **Figure 2b**, a ten-fold greater concentration of thioredoxin-ELP fusion (5  $\mu$ M versus 0.5  $\mu$ M for the standard assay described in Methods) was added and the unlabeled antibody was omitted. An equilibrium model of binding predicts that 96% of the labeled antibody should be bound at this thioredoxin concentration, assuming a  $K_D$  of  $1 \times 10^7$  M (independently determined by BIAcore analysis). However, the fraction of total fluorescence observed in the pellet is 48.4% (when corrected for pellet background fluorescence). We believe that the residual background fluorescence in the supernatant is from contaminating mouse IgG antibodies, which are present in mouse ascites fluid from which the anti-thioredoxin monoclonal antibodies are Protein-A affinity purified. This is supported by previous experiments in which residual antibodies, which remained in the supernatant after one round of capture using thioredoxin-ELP concentrations theoretically sufficient for complete capture, were not captured in a subsequent experiment by a large molar excess of thioredoxin-ELP fusion protein. This result indicates that these residual antibodies do not bind thioredoxin. Hence, if the pelleted fraction contains 96% of the anti-thioredoxin antibodies, then the fraction of contaminating antibodies which do not bind thioredoxin is 49.5%, which represents the background fluorescence for the supernatant fraction of the immunoassay.

A calibration curve for the immunoassay is shown in **Figure 3**. On the X-axis, the analyte concentration is represented by the ratio of analyte concentration (unlabeled



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assay output signal is the ratio of the fluorescence intensity of the pellet fraction to the total fluorescence intensity (i.e., the summed fluorescence intensities of the pellet and the supernatant). The background fluorescence was subtracted from the intensities for both the supernatant and pellet. Because the fluorescence intensity is quantitatively related to the concentration of the labeled anti-thioredoxin antibody, this ratio is the fraction of labeled antibody that binds to the thioredoxin-ELP fusion and is precipitated.

Qualitatively, the curve indicates that as the fraction of unlabeled antibody increases, the fraction of labeled antibody bound decreases, as expected for a competitive binding assay. Conversely, as the fraction of unlabeled antibody decreases, the fraction of labeled antibody bound increases, approaching a maximum where no competing unlabeled antibody is bound. The data exhibits a near linear relationship ( $R^2 = 0.993$ ). However the shape of the curve could be non-linear under other immunoassay conditions, as described below. Once generated, the calibration curve can then be used to quantitatively determine the concentration of unlabeled antibody in unknown samples.

Two primary parameters by which any immunoassay is judged are sensitivity and dynamic range. This competitive capture assay has a maximum sensitivity when the concentration of unlabeled analyte is equal to that of the competing labeled antibody (i.e., 0.5 on the X-axis in **Figure 3**). As the concentration of unlabeled analyte is decreased or increased from this point (e.g., moving on the calibration curve left or right from center), the interval for each successive decade in absolute concentration of analyte decreases, thereby reducing signal sensitivity. In this particular case, the immunoassay's maximum sensitivity was focused on an analyte concentration of 0.5  $\mu\text{M}$ , determined by the selection of that same concentration for the FITC-labeled antibody. This center of sensitivity could be shifted, or focused, by simply varying the labeled antibody concentration. The range over which this shift could be achieved would be limited in higher concentrations only by the supply of reagents and in lower concentrations by the antibody's binding affinity. The antibody affinity, and hence the lower limit for this particular assay, are as yet undetermined. However, preliminary calculations indicate that the antibody affinity is on the order 0.1  $\mu\text{M}$ , yielding a lower limit for the concentration of analyte of approximately 0.01  $\mu\text{M}$ .

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therefore significantly lower concentrations could be measured with immunoassays utilizing higher affinity binding pairs.

The dynamic range of a particular immunoassay (that is, the useful range on either side of a given center of sensitivity) is determined primarily by the sensitivity of the fluorimeter or other reporter detection instrumentation. For the data in **Figure 3**, the range for the most highly sensitive concentration measurements spans roughly two orders of magnitude (ranging from 50 nM to 5  $\mu$ M). At least two additional orders of magnitude (one on each end of this range) could be determined with a sensitivity roughly 10% of that for the central region, for a total range of 5 nM to 50  $\mu$ M. The absolute upper and lower limits of the useful range about the center of sensitivity have not yet been determined experimentally, but may even exceed this preliminary estimate of four orders of magnitude.

Just as the selection of the labeled antibody concentration has important ramifications on the center of sensitivity, equilibrium models of competitive binding reveal that the selection of antigen (thioredoxin-ELP) concentration critically affects the shape of the calibration curve, as alluded to earlier. Specifically, if the antigen concentration is higher than the labeled antibody concentration, then the curve takes on a non-linear shape with a plateau at lower unlabeled antibody concentrations giving way to a rapid drop-off in signal at higher antibody concentrations. This results in a significant decrease in the dynamic range of the assay. On the other hand, as the antigen concentration becomes smaller than the labeled antibody concentration, the curve takes on a more linear shape which is also accompanied by an overall reduction in fraction of total antibody bound and captured, and hence a reduction in signal sensitivity. The optimum conditions for the assay, therefore, requires equal concentrations of antigen and labeled antibody, which produces both a nearly linear curve and maximum sensitivity. However, this parameter could be varied depending on the requirements of a particular immunoassay, with high antigen concentrations used to achieve very high sensitivity over a small dynamic range, or conversely low antigen concentrations used to achieve a very broad range with reduced overall sensitivity.

The precipitation of the ELP-antibody-antigen complex can be induced by

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combination of both. The  $T_i$  of a given ELP construct is decreased as the ionic strength of the solution is increased. Therefore, aggregation may be induced isothermally by increasing the ionic strength such that the ELP  $T_i$  is depressed below the solution temperature. In addition, the ELP phase transition can also be induced in  
5 modified ELP sequences containing appropriate residues by known changes in pH, or in response to chemical or biochemical modification (e.g., phosphorylation). Our claim for this immunoassay therefore covers any physical, chemical, or biological phenomenon that can induce a phase transition in an ELP sequence or its derivatives.

Selection of the means of precipitation depends primarily on ease of use,  
10 equipment available, and avoidance of potential deleterious effects on the reactants. For example, high saline concentrations could interfere with antibody binding, while high temperatures could cause protein denaturing. However, sufficient flexibility exists in the design of the ELP  $T_i$  that gentle conditions for precipitation should be obtainable for any prospective application.

15 The ELP-precipitation immunoassay method presented here could be used in a number of different configurations or formats known to those skilled in the art. Flexibility in this assay arises from the following factors, which can be implemented in any combination: either one of the binding partners can be attached to the ELP, either one of the binding partners can be reporter-labeled, and either one of the  
20 binding partners can be the analyte. Flexibility can be further expanded by the use of a secondary antibody ("Indirect assay") or an antibody with a non-overlapping epitope ("Sandwich"). These configurations simplify the initial immunoassay reaction and eliminate the need for labeling of the reagent (which may otherwise reduce affinity), but require a secondary incubation and precipitation step.

25 The only limitation in these configurations is that the antibody cannot be both fused to an ELP and labeled with a reporter because, in this configuration, no reporter could ever be captured. Another requirement for all configurations is that for quantitative detection, only the analyte (and not its affinity partner) can occur in the unknown sample.

30 Selection of a particular configuration would depend primarily upon the availability of a gene for one of the binding partners. The availability of sufficient

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exist for which the analyte can be previously unknown. This could be useful, for example, in screening libraries of engineered antibodies for molecules specific to a particular antigen.

This immunoassay is also amenable to the measurement of multiple analytes in a single sample using a set of analyte-binding proteins, each of which is specific to an analyte of interest. Detection of each analyte can be achieved by two alternative strategies. In the first approach, each analyte-binding protein is attached to a distinct reporter (e.g., fluorophores with unique excitation and emission properties). The assay would be performed identically to that for a single analyte, and independent detection of each analyte would be simultaneously achieved by monitoring the unique spectroscopic signal for each reporter.

The alternative approach, a sequential detection scheme, would utilize fusion of each antigen or antibody to ELPs with different  $T_t$ 's. The non-covalent complexes formed would be sequentially and independently precipitated, thereby allowing separate measurement of each analyte. This approach requires ELPs with distinct, non-overlapping  $T_t$ 's, which can be achieved by a combination of varying the identity of the guest residue and by varying the ELP molecular weight. We have previously demonstrated the feasibility of this approach by the synthesis of ELPs of varying chain length exhibiting different  $T_t$ 's, and we can further expand upon the available range of  $T_t$ 's available by producing ELPs with different compositions.

The genetic attachment of the ELP provides several key advantages as compared to chemically-conjugated, synthetic temperature-sensitive polymers outlined in patent WO 86/06492. First, this method of synthesizing the thermally precipitating reagent is technically simpler and less costly, with no need for separate and often complex synthesis of the polymer nor for a subsequent chemical conjugation step to couple the immunoassay reactant to the thermally-responsive polymer. Second, the genetically-produced ELP is monodisperse, whereas production of synthetic polymers results in a population of molecules with varying chain lengths. This heterogeneity may adversely affect the performance of the immunoassay by, for example, shifting or broadening the range of temperatures over which the phase transition occurs. Finally, genetic fusion allows absolute control over the number (typically one) and location of

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conjugation sites located throughout the primary amino acid sequence of the protein, resulting in varied numbers and diverse locations of attached polymers. If any of these conjugation sites are near the binding site, reduction of affinity or complete blockage of binding results, thereby adversely affecting the performance of the immunoassay. Furthermore, chemical conjugation of protein and polymer is inefficient and incomplete, whereas genetic ELP incorporation insures that every molecule has exactly the same structure and properties.

In summary, the immunoassay described herein provides a novel method for reactions in which at least one of the binding partners is a recombinant protein. It has important advantages over similar methods in terms of simplicity and precision of reagent production. It is flexible and can be utilized in a variety of configurations, and can be readily automated. Finally, it provides high sensitivity over a wide range of medically-relevant analyte concentrations.

15

### EXAMPLE 3

#### Sensor Regeneration with a Thioredoxin-ELP Fusion Protein

This examples shows that an environmentally-responsive polypeptide tag can be introduced into a model protein by genetic engineering, that the polypeptide tag allows the fusion protein to adsorb to a hydrophobic surface, and that the fusion protein can be desorbed from the surface by altering the solvation of the protein in response to an environmental signal, thereby allowing regeneration of the surface back to the protein-free substrate. This approach is generic for the following reasons: first, the introduction of enviromentally-triggered properties in a target protein simply involves gene-level N or-C-terminal fusion of the peptide codons into a cloned or synthetic gene, which is easily achieved by standard molecular biology manipulations. Second, fusion proteins containing this polypeptide tag are rendered environmentally-responsive, and this appears to be a fairly general phenomena. Finally, the polypeptide tag can be chemically conjugated to molecules that are not genetically-encodable, thereby creating an environmentally-responsive bioconjugate that can be reversibly adsorbed and desorbed by small changes in solution conditions.

### A. Experimental Details

**Solution Inverse Transition.** The optical absorbance at 350 nm of the thioredoxin-ELP fusion protein was monitored in the 4-60 °C range on a Cary Bio-300 UV-visible spectrophotometer, equipped with a multi-cell peltier temperature  
5 controller. The  $T_1$  was determined from the midpoint of the change in optical absorbance at 350 nm due to aggregation of ELP or thioredoxin-ELP fusion protein as a function of temperature at a heating or cooling rate of 1-1.5 °C min<sup>-1</sup>.

**Substrate Preparation:** Gold substrate were prepared by thermal evaporation of 50 Å Cr on a silicon wafer (for ellipsometry) or glass slide (for BIACORE instrument  
10 measurements) followed by 500 Å gold. The gold substrate were cleaned in a 1:1:3 solution of NH<sub>4</sub>OH:H<sub>2</sub>O<sub>2</sub>:H<sub>2</sub>O and incubated overnight in a 1mM solution of hexadecanethiol in ethanol to form a self-assembled monolayer (SAM) on gold. The SAMs were sonicated in ethanol for 1 min, dried under nitrogen and used immediately thereafter. Advancing water contact angle of the SAMs were ~100°. for  
15 the hydrophobic SAM of hexadecanethiol.

**In situ Ellipsometry.** The SAMs were mounted on the ellipsometer stage in a cuvet and the cuvet was filled with 50 mM phosphate, pH 7.4, 1 M NaCl. The polarizer and analyzer angle of the thiol-functionalized gold substrate were measured in buffer and converted to ellipsometric parameters ( $\psi$ ,  $\Delta$ ). Next, a concentrated stock  
20 solution of thioredoxin/ELP fusion protein was pipetted into the cuvet to a final concentration of 1  $\mu$ M. The time course of adsorption of the fusion protein on SAM-functionalized gold substrates was examined by *in situ* ellipsometry as the ELP underwent a hydrophilic-hydrophobic transition as the temperature was raised from 15 -40 °C at ~1 °C min<sup>-1</sup>. The ellipsometer cuvet was heated and cooled by an  
25 immersion coil connected to a thermally programmable (-20-100° C) recirculating water bath. The *in situ* temperature of the buffer in the cuvet was measured by a thermocouple at each time point for which ellipsometric parameters were measured. The polarizer and analyzer angles were monitored through the thermal ramp on the upward and downward cycle and were used in a nonlinear regression simulation  
30 program to obtain thickness and complex refractive indices for the silicon substrate.

**BIACORE™ Instrument Analysis:** Typically, a gold-coated glass slide was mounted in an empty BIACORE™ sensor cartridge using water-insoluble double-sided sticky tape. The sensor cartridge was docked into a BIACORE X™ instrument system, and a system check was performed to ensure the absence of leaks in the fluid path and minimal baseline drift. The cartridge was then removed from the instrument and cooled to low temperature (approximately -20 °C). The sensor surface was incubated with 1 µM thioredoxin-ELP fusion protein in 2 M NaCl at ~4° C, warmed to room temperature to allow the fusion protein to undergo the phase transition, and adsorbed to the sensor surface for 5 min. Excess protein was washed away with buffered 2M NaCl at room temperature and the sensor cartridge was then reinserted into the BIACORE X™ instrument, which was maintained at 35 °C. All buffers used for BIACORE™ instrument measurements were 50 mM potassium phosphate, pH 7.4 of varying NaCl concentrations.

## B. RESULTS AND DISCUSSION.

**Design of ELP Carrier.** Based on previous studies that have delineated the effect of varying the identity of the fourth, guest residue (X) in the repeat unit, and its mole fraction on the  $T_1$ , a gene was synthesized encoding an ELP sequence with guest residues Val (V), Ala (A), and Gly (G) in the ratio 5:2:3 in accordance with the procedures described in Example 1 above to provide a solution  $T_1$  in water of ~37 °C. By adjusting the sequence length, as well as its composition, we can design sequences that undergo the inverse transition at any temperature between 0 and 100 °C. The ELP-1 mer (50 amino acids) was oligomerized to produce a library of ELP oligomers at the gene level. The 18-mer was chosen as a carrier polypeptide in the first generation expression vector, based on its experimentally-observed  $T_1$ .

**Inverse Transition of Thioredoxin-ELP Fusion Protein in Solution.** The inverse transition of thioredoxin-ELP 18-mer fusion protein (25 µM fusion protein in 50 mM phosphate, 1 M NaCl) as a function of temperature (1.5 °C min<sup>-1</sup> heating rate) is shown in **Figure 4**. With the exception of a higher concentration of fusion protein, these experimental conditions are identical to those used for surface regeneration. As the temperature was raised, the turbidity of the solution, spectrophotometrically

- 30 -

a maximum value, due to aggregation of the thioredoxin-ELP fusion protein. The inverse transition temperature ( $T_I$ ) is defined as the midpoint of the spectrophotometrically observed transition. The experimentally-observed  $T_I$  of the thioredoxin-ELP fusion was 25 °C, which was identical to that of the ELP with no fusion partner (results for ELP not shown), demonstrating that the inverse transition of the ELP was not affected by conjugation to thioredoxin. This process was reversible with slight hysteresis, and the aggregated fusion protein could be resolubilized completely by lowering the temperature below the  $T_I$ . The thioredoxin control exhibited no change in absorbance with increasing temperature. We also observed complete retention of thioredoxin activity after several rounds of thermal cycling. These results validate our first hypothesis, that the inverse transition of the ELP sequence is retained upon its expression in a fusion protein, and that the ELP tag does not compromise the activity of the target protein, which is critical for biosensor applications.

**Surface Binding and Regeneration of Thioredoxin-ELP Fusion Protein.** This experiment was designed to investigate the hypothesis that desolvation of the ELP sequence upon raising the temperature above its  $T_I$  will drive a protein to the surface via hydrophobic interactions between the desolvated, hydrophobic ELP sequence and the hydrophobic surface. Subsequently, upon lowering the temperature below the  $T_I$ , the ELP sequence solvates and becomes hydrophilic, which should lead to spontaneous desorption of the ELP fusion protein from the surface.

The adsorption of the fusion protein on the hydrophobic SAM was examined by *in situ* ellipsometry as the ELP underwent a hydrophilic-hydrophobic transition in 50 mM phosphate, 1 M NaCl as the temperature was raised from 15 -40 °C at a rate of 1 °C min<sup>-1</sup>. The polarizer and analyzer angles were monitored through the temperature ramp. **Figure 5** shows the change in thickness of the protein adlayer as a function of temperature, calculated by nonlinear regression using the measured values of the polarizer and analyzer angles. The increase in adlayer thickness with increasing temperature clearly shows that as the thioredoxin-ELP fusion protein undergoes its inverse transition, it adsorbs to the hydrophobic SAM during the upward thermal ramp. The observed inverse transition of the ELP on the hydrophobic surface is



- 31 -

hydrophobic surface above the transition temperature. Upon lowering the temperature to  $\sim 20^{\circ}\text{C}$ , the adsorbed thioredoxin-ELP fusion protein desorbs from the surface. Desorption is essentially complete as seen by the  $< 5\text{ \AA}$  change in surface thickness after one thermal cycle.

5        In separate experiments, we have established that the adsorbed protein completely desorbs from the surface, if the maximum temperature in the cuvet is kept below  $40^{\circ}\text{C}$ . This process can be repeated several times with virtually no irreversibility. A control experiment with thioredoxin alone, showed little adsorption of thioredoxin in the time scale of the experiment, and all of it was irreversible. This  
10        experiment demonstrates the reversible adsorption/desorption of an ELP fusion protein in response to a thermal stimulus.

**Analyte Binding and Surface Regeneration by Surface Plasmon Resonance.**

The objective of this set of experiments was to demonstrate that adsorbed thioredoxin-ELP fusion protein can bind to an analyte, anti-thioredoxin mAb, and that the  
15        antibody-fusion protein complex can be desorbed after binding. This objective can be achieved if the following can be demonstrated:

1) Thioredoxin is exposed to the bulk after immobilization of the ELP-thioredoxin fusion protein.

2) A monoclonal antibody against thioredoxin can bind to the surface-immobilized thioredoxin.  
20

3) The thioredoxin-ELP/antibody complex remains immobilized above the phase transition over time.

4) Thioredoxin-ELP/antibody complex can be desorbed from the surface upon lowering the temperature below the phase transition temperature.

25        The BIACORE<sup>TM</sup> device divides the sensor surface into two channels which can be addressed individually. This allows use of one channel as a reference, and all signals presented here are difference signals between channel 1 (measurement) and channel 2 (reference). As shown in **Figure 6**, the bare surface [A] has a signal difference between channel 1 and channel 2 of about 530 Response Units (RU). This  
30        is due to small variations in substrate preparation. At time [1] the sensor surface, to

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found, which may be due to the insertion process. The reference channel 2 is then rinsed with pure water for 5 minutes [2] which removes the adsorbed thioredoxin-ELP fusion protein from the sensor surface in this channel. The signal difference [C] of about 3300 RU corresponds to the amount of adsorbed thioredoxin-ELP fusion protein in channel 1. At high salt concentration, 2 M NaCl in the running buffer, the signal is stable, but antibody binding is inefficient. Therefore the salt concentration in the running buffer was reduced to 1 M NaCl [3], which leads to a slow desorption [D] of about 20% of the protein in about 1000 sec. The stable desorption rate is about 0.4 RU/sec.

10       **Anti-thioredoxin binding.** Channel 1 is selectively incubated with 50 µl anti-thioredoxin mAb (1:10 dilution) in running buffer [4]. The signal increases by about 1600 RU [E] indicating strong antibody binding. Upon completion of binding, only the time-dependent desorption at a rate of 0.4 RU/sec is observed.

15       **Desorption.** The sensor temperature can be changed in the BiacoreX, and although the temperature change is not linear with time, the temperature is measured at the sensor surface, and can be monitored as a function of time. Upon lowering the temperature [5] from 35 °C to 18 °C the signal decreases [F] to approximately the same level (400 RU) as the bare sensor surface. **Figure 7** shows the desorption process as a function of temperature.

20       These experiments demonstrate that a thioredoxin-ELP fusion protein adsorbs to a hydrophobic surface and can be desorbed completely by a small change of environmental conditions. The observation that after adsorption of the fusion protein, thioredoxin binds to an anti-thioredoxin mAb, suggests that the water soluble thioredoxin exposed to the bulk and accessible to its antibody. The immobilized complex is stable at the surface; in a separate experiment with a running buffer containing 2 M NaCl, after mAb binding, the SPR was stable to within 0.08 RU/sec over several hours, though the affinity for antibody binding is reduced at these salt concentrations (data not shown). Together, these independent experiments show that the noncovalent complex of fusion protein and antibody can be immobilized on the surface with high stability. Finally, the complex can be desorbed completely from the

25  
30

- 33 -

These data indicate the feasibility of creating a regenerable surface with the desired sensing specificity in accordance with the present invention. We believe that our approach to sensor regeneration is powerful and generic. This is because an analyte-binding protein for biosensor applications can easily be produced as a fusion with an ELP tag using standard molecular biology methods with no loss of functional activity. For other molecules which cannot be produced as a genetically-encoded fusion with an ELP, including proteins for which a cloned or synthetic gene is unavailable, the ELP tag can be chemically or biochemically-conjugated to the target molecule. Creating a reversible biosensor surface then only requires a hydrophobic surface, which is also easily achieved by a variety of surface functionalization methods, including SAMs on gold, silane modification of glass or oxidized silicon, or any other method that provides a hydrophobic surface. Combined, the environmentally-triggered, reversible interaction of an ELP fusion protein and a hydrophobic surface allows facile regeneration of biosensors.

15

#### EXAMPLE 4

##### Targeted Delivery of Therapeutics to Solid Tumors

##### By Thermally Responsive Polymers

Because targeting is necessary for efficient tumor localization of systemically-delivered therapeutics, we propose a thermal targeting scheme, where a thermally-responsive drug carrier precipitates in regions of elevated temperature.

Polymers that undergo an inverse temperature transition are useful as drug carriers for thermal targeting because they are soluble in aqueous solutions below their inverse transition temperature ( $T_1$ ), but when the temperature is raised above their  $T_1$ , they undergo a narrow phase transition within a 2-3 °C range, leading to extensive desolvation and aggregation. Elastin-like protein (ELP) biopolymers [D. Urry, *J. Prot. Chem.* **7**: 1-34 (1988); D. Urry, *Prog. Biophys. Molec. Biol.* **57**: 23-57 (1992)] and copolymers of poly(N-isopropylacrylamide) (poly(NIPAAm)) [H. Schihld, *Protg. Polym. Sci.* **17**: 163-249 (1992); C. Chiklis and J. Grasshof, *J. Polym. Sci. Part A-2* **8**: 1617 (1970); L. Taylor and L. Cerankowski, *J. Polym. Sci. Polym. Chem.* **13**: 2551 (1975); Y. Takei, *Bioconj. Chem.* **4**: 42 (1993)] are two polymer

- 34 -

temperature. ELP biopolymers can be designed to exhibit a  $T_i$  within a  $<2^\circ\text{C}$  range between  $0-100^\circ\text{C}$  by specifying the identity and fraction of the fourth residue [D. Urry et al., *J. Am. Chem. Soc.* **113**: 4346 (1991)]. Chemically synthesized copolymers of NIPAAm and alkylacrylamides, display a similar phase transition, the midpoint of which can similarly be tuned by varying the fraction of the NIPAAm monomer. To date, neither of these polymers have been utilized as radionuclide carriers for targeted drug delivery by hyperthermia.

#### A. METHODS

Gene synthesis, oligomerization, protein expression, purification, Poly (NIPAAm) synthesis and inverse transition temperature characterization are described in Example 1 above.

**Conjugation of Fluorophore.** Approximately 11 milligrams of thrombin-cleaved ELP-15 mer or the poly(NIPAAm/AAm) copolymer were resuspended in 1 ml 100 mM sodium bicarbonate buffer, pH 8.34. 40  $\mu\text{l}$  of Rhodamine Red-X<sup>TM</sup> succinimidyl ester (10 mg/ml in DMF) was added and incubated with gentle agitation for 2 hours at room temperature. After incubation, the sample was centrifuged at 10,000 g for 5 min. to remove insoluble matter. The supernatant was then thermally purified by adding NaCl to 500 mM, heating to  $35^\circ\text{C}$ , and centrifugation at  $T \geq 35^\circ\text{C}$  at 10,000 g for 5 minutes. The supernatant was removed, the pellet was resuspended in cold PBS, and centrifuged cold. The resolubilized conjugate was retained. The thermal purification was repeated twice. Conjugation efficiency for the ELP 15-mer was determined by BCA total protein assay and by rhodamine absorption at 570 nm.

**Tumor Vascular Preparation.** A small volume ( $\sim 0.1\text{ mm}^3$ ) of tumor tissue (human ovarian carcinoma; SKOV-3) was implanted in the dorsal skin flap window chamber in nude mice. The implanted tumors were used 10-15 days after surgery, when the tumor in the window chamber was 2-3 mm in diameter. The implanted tumors in the window chamber can be placed under a microscope so that the injected material of interest can be directly visualized in the vascular and interstitial compartments and its concentration can be directly measured.

**Experimental Procedure for Mouse Window Chamber Technique.** In

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cannulation, the animal was placed on a temperature-controlled microscope stage to maintain normal body temperature throughout the experiment. The tumor in the window chamber was observed with a 20X objective. A region of the preparation was selected such that blood flow appeared to be normal and did not have any other blood vessels below it. Images of the selected region were recorded by a SIT camera connected to a S-VHS recorder under epifluorescent illumination before injection of the ELP-Rhodamine conjugate. After injection of the conjugate ( $t = 0$ ), the image from the selected region was recorded every 2 min. for 5 sec over a duration of 40 min. The videotape was analyzed by image processing software (NIH Image) on an IBM-compatible-PC. At any time, the light intensity of the entire tissue area ( $I_t$ ), and a representative vascular region ( $I_v$ ) and interstitial region ( $I_i$ ) were analyzed to provide the time dependence of  $I_v$  and  $I_i$ .

## **B. RESULTS**

**Design, Synthesis, Purification, and Characterization of ELP Biopolymer and Poly(NIPAAm/AAm) Copolymer.** The transition temperature of an ELP biopolymer is strongly influenced by the hydrophobicity of the guest residue (X) and its mole fraction  $f_x$ . Therefore, by varying the identity of guest residues, and their mole fraction, ELP copolymers can be designed to exhibit a predetermined inverse transition temperature in a 0-100 °C range. Based on these previous studies, a synthetic gene for the monomer, which encodes 10 VPGXG repeats, was designed to incorporate guest residues: X = valine (V), alanine (A), and glycine (G) in the ratio 5:2:3 to achieve a  $T_1$  of 42 °C. Standard molecular biology procedures were used to construct the synthetic gene of the ELP monomer, and to oligomerize the gene to create a library ranging from 1 to 18-mer where each monomer contains 50 residues.

In initial studies, the ELP 6-, 9-, 12- and 18-mers were expressed in *E. coli* in a modified pET-32b expression vector as a fusion with thioredoxin. Each fusion protein retained the reversible phase transition of the ELP sequence, which allowed one-step thermal purification of the thioredoxin-ELP fusion from all other soluble *E. coli* contaminants. The amino acid sequence of the fusion protein has a recognition site specific to thrombin, located between thioredoxin and the ELP sequence.

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Thermal precipitation of the ELP was then used to purify the ELP from other proteins. Because the thermally-induced aggregation of ELP biopolymer is completely reversible *in vitro*, the ELP was then solubilized for subsequent experiments by simply lowering the temperature below its  $T_1$ . The observation that the thioredoxin  
5 fusion protein containing a C-terminal ELP sequence also exhibits an inverse transition indistinguishable from the ELP sequence alone is exciting, because it indicates that thermal targeting will also be applicable to the delivery of protein therapeutics to tumors.

The thermal behavior of the ELP 6, 9, and 18-mer (MWs = 24.3 kDa, 35.9 kDa,  
10 and 71 kDa, respectively) in PBS ranged from 58.7 °C to 35.1 °C is shown in **Figure 8**, and reveals a strong inverse relation between MW and  $T_1$ , which is consistent with previous observations of Urry and colleagues for ELPs with MWs < 100 kDa. Interpolation of these results suggested that an ELP 15-mer (MW ~50 KDa) would allow the target  $T_1$  of 40-42 °C to be achieved with the current ELP composition,  
15 containing guest residues Val:Ala:Gly in a 5:2:3 ratio.

The ELP 15-mer was then synthesized and purified in mg quantities. **Figure 9** displays the thermal behavior of the ELP 12-mer in physiological saline. The  $T_1$  of the ELP 15-mer, defined as the midpoint of the experimentally-observed inverse transition, is 42.5 °C. The inverse transition of this polymer occurs over a narrow, <3  
20 °C range, which is important for hyperthermic targeting. While the first generation ELP 15-mer construct will be useful for *in vivo* radionuclide delivery, it would be desirable to have in hand additional constructs with MWs in the 10-30 kDa range with a  $T_1$  of 40 °C, in order to explore the relationship between ELP MW and radionuclide biodistribution. Based on previous studies by Urry *et al.*, the Val:Ala:Gly ratio is  
25 decreased to 5:1:1 to yield a polymer of 25 kDa MW and a  $T_1$  of 40 °C and is useful in the same manner as described above.

The thermal behavior of the poly(NIPAAm/AAm) copolymer is also shown in **Figure 9**, and clearly demonstrates that the inverse transition occurs in a narrow, 2°C range, and that the  $T_1$  is 41 °C. The copolymer contains one amine end group per  
30 polymer chain for conjugation of amine-reactive radionuclides. Future rounds of

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to monomer. If required, after synthesis the polymers can be fractionated by size to provide polymers with a specified MW and polydispersity.

**Characterization of Rhodamine-Polymer Conjugates.** The thermal behavior of the ELP 15-mer and its conjugate with Rhodamine is shown in **Figure 10**. The conjugation of Rhodamine to the ELP 15-mer results in a decrease in the  $T_1$  from 43 °C to 40 °C. This is consistent with the increased hydrophobicity of the conjugate, which should result in a decrease in the observed  $T_1$ . Similarly, conjugation of Rhodamine to the poly(NIPAAm/AAm) copolymer also results in a decrease in the  $T_1$  of the conjugate, but the magnitude ( $\sim 1$  °C) is slightly smaller than that observed for the ELP.

**Fluorophore Targeting Study: Dorsal Skin Flap Window Model.** Equal amounts of Rhodamine-labeled ELP-12 mer (1.1 mg, 200  $\mu$ l) or poly(NIPAAm) were injected into nude mice bearing subcutaneous ovarian cancer tumors (SKOV-3) at normothermic and hyperthermic temperature into two animals. In one experiment, the window chamber was maintained at normothermic temperature (34° C), while in the second experiment, the window chamber containing the implanted s.c tumor was heated to 42° C. Results from these two experiments are shown in **Figures 11a** and **11b**. Over a period of 40 min., the fluorescence in the vasculature remains constant and approximately equal at both 34 °C and 42 °C. However, a significant difference is observed in the interstitial concentration of the Rhodamine-ELP conjugate. At 42° C, the fluorescence intensity is four-fold higher than at 34° C. These results clearly indicate that the ELP is able to extravasate out of the tumor endothelium and preferentially accumulates in the interstitium at 42 °C as compared to 34 °C. **Figure 12** also shows the qualitative difference between the behavior of the ELP conjugate at the two different temperatures. **Figure 12a** is a captured image of the tumor under hyperthermic conditions (42° C), and **Figure 12b** is a fluorescence image for the normothermic control (34° C). At 42° C, fluorescent aggregates are clearly visible in the tumor microvasculature, whereas in the control, normothermic animal, no such aggregates are visible, indicating that the ELP conjugate selectively precipitates in the tumor microvasculature at 42° C. These results are consistent with our in vitro

Figure 12a shows the fluorescence image of the tumor at 42° C. The Rhodamine-ELP conjugate was

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39° C. Similar results were also obtained for a Rhodamine-poly(NIPAAm/AAm) copolymer (results not shown).

### C. CONCLUSIONS

This example demonstrates that:

5 (1) Two different classes of polymers have been synthesized that display thermally-responsive behavior in the range of 40-42 °C in physiological saline, a temperature range that is clinically relevant for hyperthermia. The two polymers are (a) an artificial polypeptide, based on a pentapeptide repeat found in elastin, and (b) a poly(NIPAAm/AAm) copolymer. The thermally-responsive behavior is manifested  
10 as a solubility-insolubility transition, where the polymer is soluble below the  $T_1$ , and insoluble above the  $T_1$ .

(2) In vitro, we have also demonstrated that this transition is reversible, so that the polymers can be cycled between their soluble and insoluble states as a function of temperature.

15 (3) For the ELP polymers, we have demonstrated that upon fusion of an ELP with another protein the thermally-responsive behavior is retained, and is identical to that observed for the ELP alone. This clearly indicates that protein therapeutics can be synthesized in vivo as fusion protein with an ELP carrier, thereby obviating the need for post-expression chemical conjugation.

20 (4) Both classes of polymers have been synthesized to contain a specified number of reactive groups so as to allow conjugation of fluorophores, radionuclides, and drugs. We conjugated a fluorophore, Rhodamine, both to the ELP and to the poly(NIPAAm/AAm) copolymer. The fluorophore-polymer conjugate demonstrated a similar  $T_1$  (within 1-3 °C) of the unconjugated polymer. This decrease  
25 in the  $T_1$  after conjugation was addressed by selecting a polymer with a  $T_1$  slightly higher than optimal for hyperthermia, i.e., between 42-45 °C, so that upon conjugation, the  $T_1$  of the conjugate is then in the range of 40-42 °C.

(5) The delivery of the thermally-responsive ELP and poly(NIPAAm/AAm) copolymer was investigated with and without hyperthermia. Aggregation and



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observed in the tumor window of normothermic, control mice. Quantitative analysis of the time course of vascular and interstitial concentration of the thermally-responsive polymers showed that upon application of hyperthermia, the interstitial concentration of the ELP was enhanced by a factor of 250% in a 40 min period of  
5 hyperthermia over that observed for the normothermic control. We conclude that enhanced delivery of therapeutics can be achieved by conjugation to thermally-responsive macromolecule carriers in concert with the targeted application of hyperthermia to tumors.

The foregoing is illustrative of the present invention, and is not to be construed  
10 as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

**We claim:**

1. A method of binding a compound of interest in an aqueous solution, said method comprising the steps of:

5 (a) providing a conjugate comprising a bioelastic compound and a binding compound, wherein said binding compound specifically binds said compound of interest, and wherein said bioelastic compound has a transition temperature below which said bioelastic compound is soluble in said solution, and above which said bioelastic compound is insoluble in said solution;

10 (b) contacting said conjugate to said compound of interest in an aqueous solution so that said compound binds thereto, with said contacting step carried out at a temperature below the transition temperature of said bioelastic compound; and then

(c) raising the temperature of said conjugate to a temperature above the transition temperature of said bioelastic compound so that said conjugate separates from said aqueous solution with said compound of interest bound thereto.

15

2. A method according to claim 1, wherein said binding compound is a protein or peptide.

20 3. A method according to claim 1, wherein said binding compound is an antibody.

4. A method according to claim 1, wherein said compound of interest is a protein or peptide.

25 5. A method according to claim 1, wherein said compound of interest is an antibody.

6. A method according to claim 1, wherein said conjugate is a fusion protein.

30 7. A method useful for immunologically detecting an analyte in an aqueous solution, said method comprising the steps of:

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wherein said bioelastic compound has a transition temperature below which said bioelastic compound is soluble in said solution, and above which said bioelastic compound is insoluble in said solution;

5       (b) contacting said conjugate to said analyte in an aqueous solution so that said compound binds thereto, with said contacting step carried out at a temperature below the transition temperature of said bioelastic compound;

      (c) raising the temperature of said conjugate to a temperature above the transition temperature of said bioelastic compound so that said conjugate separates from said aqueous solution with said analyte bound thereto; and then

10       (d) detecting said analyte.

8. A method according to claim 1, wherein either said analyte or said binding compound is an antibody.

15       9. A method according to claim 7, wherein said method selected from the group consisting of direct immunoassays, indirect immunoassays, and sandwich assays.

20       10. A method according to claim 7, wherein said conjugate is a fusion protein.

      11. An article useful as a regenerable biosensor for binding a compound of interest from an aqueous solution, said article comprising:

      (a) a solid support having a hydrophobic surface formed thereon; and

25       (b) a conjugate reversibly bonded to said hydrophobic surface, said conjugate comprising (i) a bioelastic compound and a (ii) binding compound, wherein said binding compound specifically binds said compound of interest, and wherein said bioelastic compound has a transition temperature below which said bioelastic compound is soluble in said solution, and above which said bioelastic compound is insoluble in said solution; so that said biosensor may be used to bind said compound  
30       of interest at a temperature above said transition temperature, and so that said conjugate can be removed from said solid support for recycling of said article by

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12. An article according to claim 11, wherein said binding compound is a protein or peptide.

13. An article according to claim 11, wherein said binding compound is an  
5 antibody.

14. An article according to claim 11, wherein said compound of interest is a protein or peptide.

10 15. An article according to claim 11, wherein said compound of interest is an antibody.

16. An article according to claim 11, wherein said conjugate is a fusion  
15 protein.

17. An article according to claim 11, wherein said solid support is a gold  
support.

18. An article according to claim 11, wherein said solid support comprises a  
20 polymer.

19. An article according to claim 11, wherein said solid support comprises a metal.

20 25 20. An article according to claim 11, wherein said solid support comprises a semiconductor.

21. A method of recycling a used biosensor to which has been bound a compound of interest, said method comprising:

30 (a) providing a biosensor comprising a solid support having a hydrophobic surface formed thereon and a first conjugate reversibly bonded to said hydrophobic

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specifically bound thereto, and wherein said bioelastic compound has a transition temperature below which said bioelastic compound is soluble in said solution, and above which said bioelastic compound is insoluble in said solution; and then

(b) separating said conjugate with said compound of interest bound thereto  
5 from said solid support by lowering the temperature of said biosensor to below said transition temperature; and then

(c) binding a second conjugate to said hydrophobic surface, said second conjugate comprising a second bioelastic compound and a second binding compound, so that said biosensor may be reused.

10

22. A method according to claim 21, wherein said first conjugate and said second conjugate are the same.

23. A method according to claim 21, wherein said first binding compound is a  
15 protein or peptide.

24. A method according to claim 21, wherein said first binding compound is an antibody.

20 25. A method according to claim 21, wherein said compound of interest is a protein or peptide.

26. A method according to claim 21, wherein said compound of interest is an antibody.

25

27. A method according to claim 21, wherein said conjugate is a fusion protein.

31. A method for the targeted delivering of a compound *in vivo* to a selected  
30 region within a subject, said method comprising:

(a) administering a conjugate to said subject, said conjugate comprising said

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transition, wherein said polymer has a transition temperature ( $T_1$ ) greater than the temperature at which said compound is delivered; and then

(b) heating said selected region to a temperature greater than the transition temperature of said polymer, so that said compound is preferentially delivered to said  
5 selected region.

32. A method according to claim 31, wherein said polymer is a bioelastic polymer.

10 33. A method according to claim 31, wherein said polymer is a poly(N-isopropylacrylamide) polymer.

34. A method according to claim 31, wherein said compound is an imaging agent.  
15

35. A method according to claim 31, wherein said compound is a therapeutic agent.

36. A method according to claim 31, wherein said compound is a  
20 radionuclide.

37. A method according to claim 31, wherein said selected region is a limb.

38. A method according to claim 31, wherein said selected region is an organ.  
25

39. A method according to claim 31, wherein said selected region is hyperproliferative tissue.

40. A method according to claim 31, wherein said selected region is a solid  
30 tumor.

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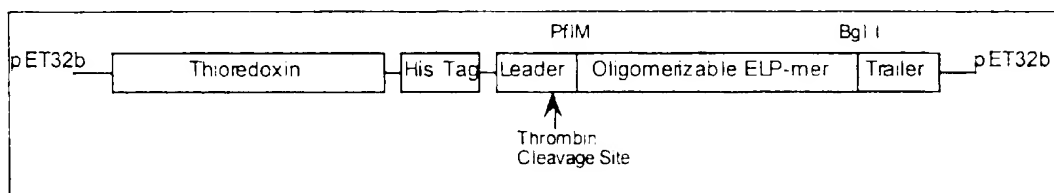


Figure 1

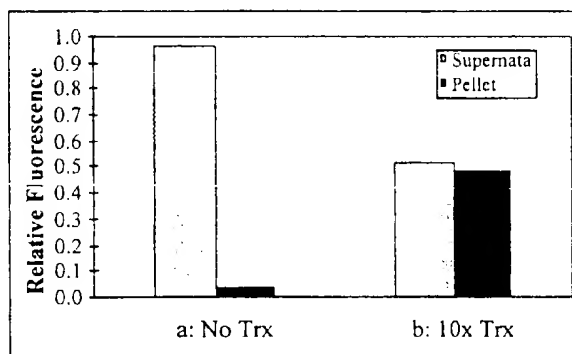


Figure 2

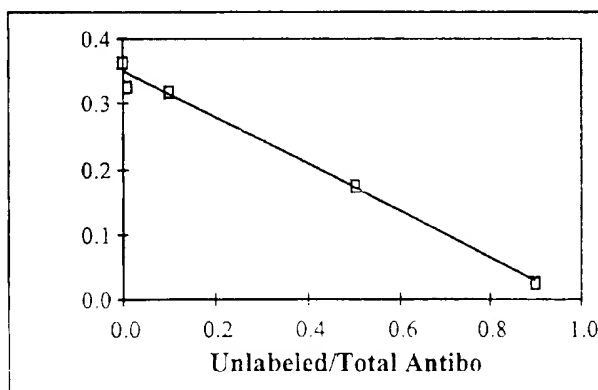


Figure 3

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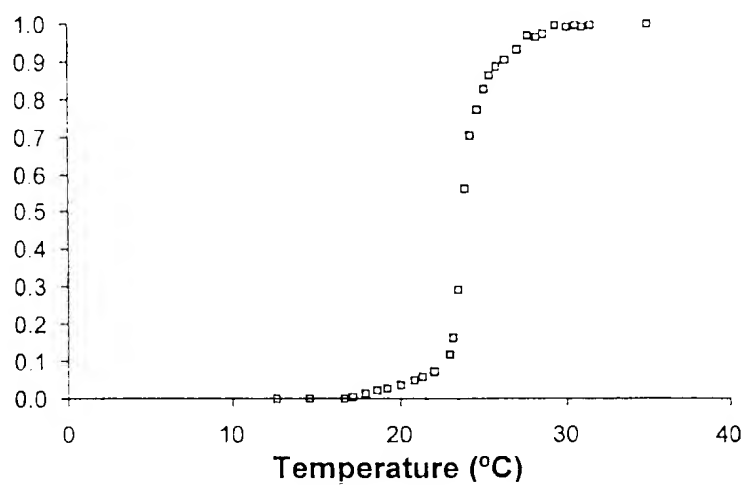
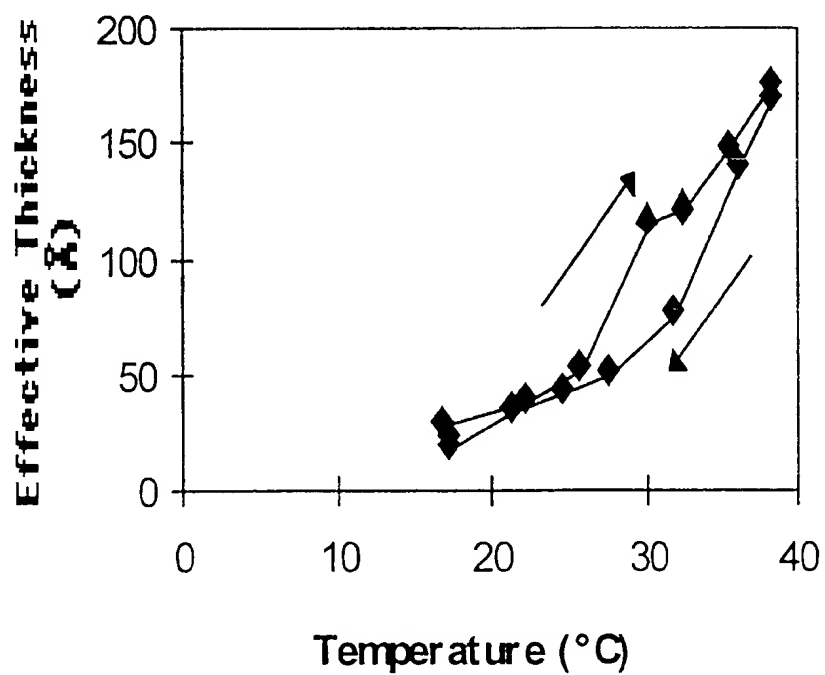


Figure 4





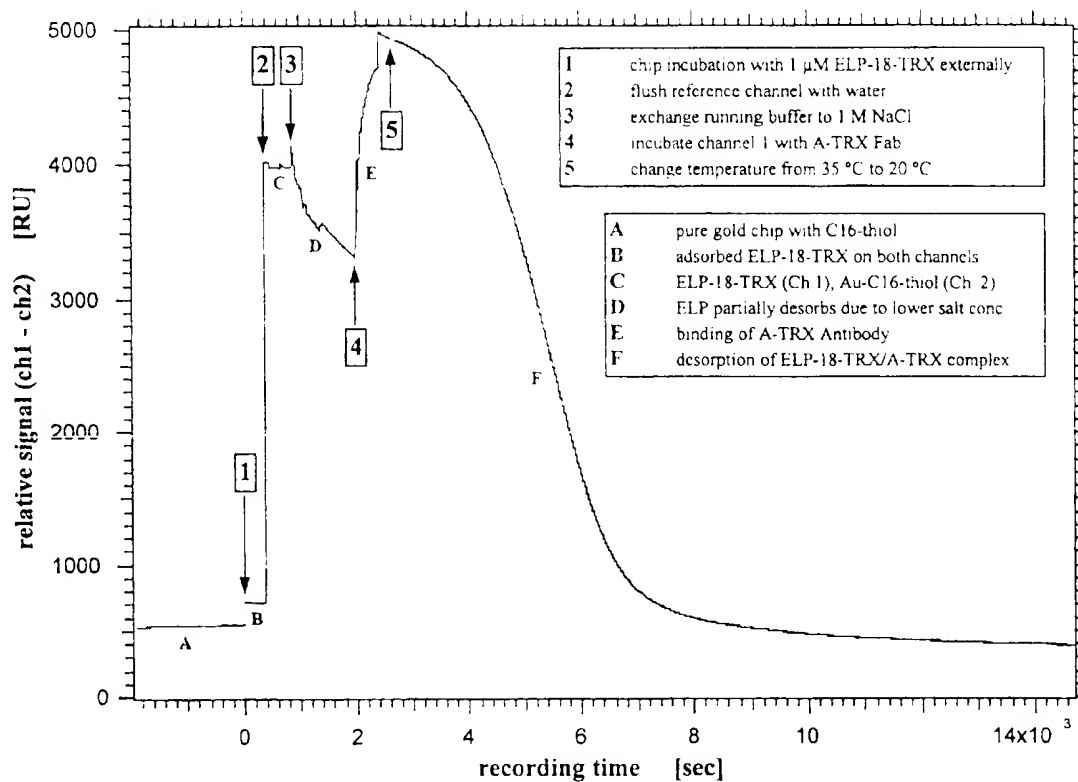


Figure 6

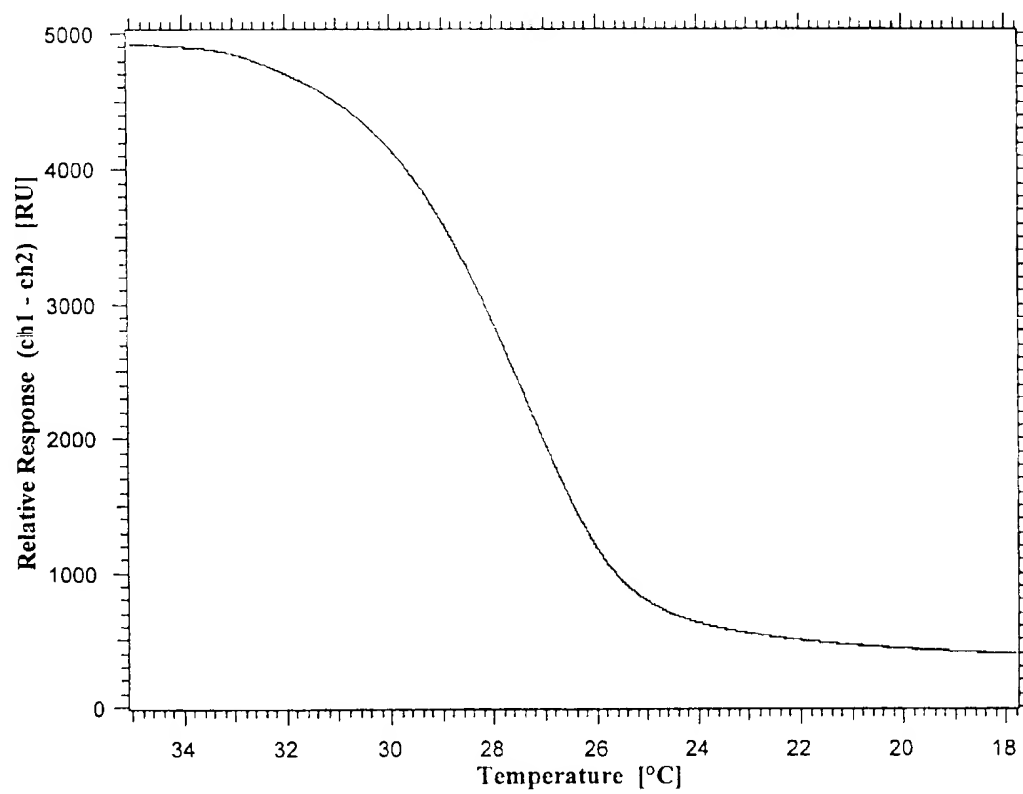


Figure 7

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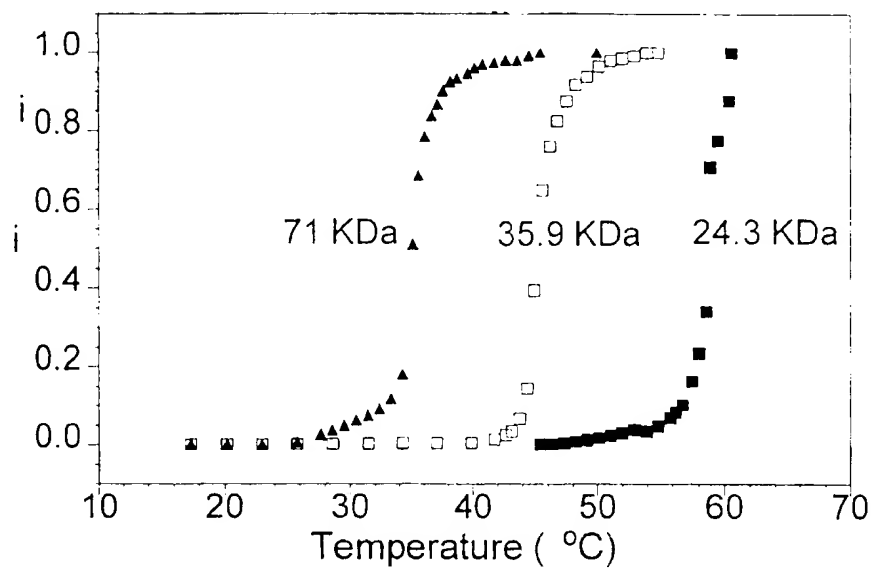


Figure 8

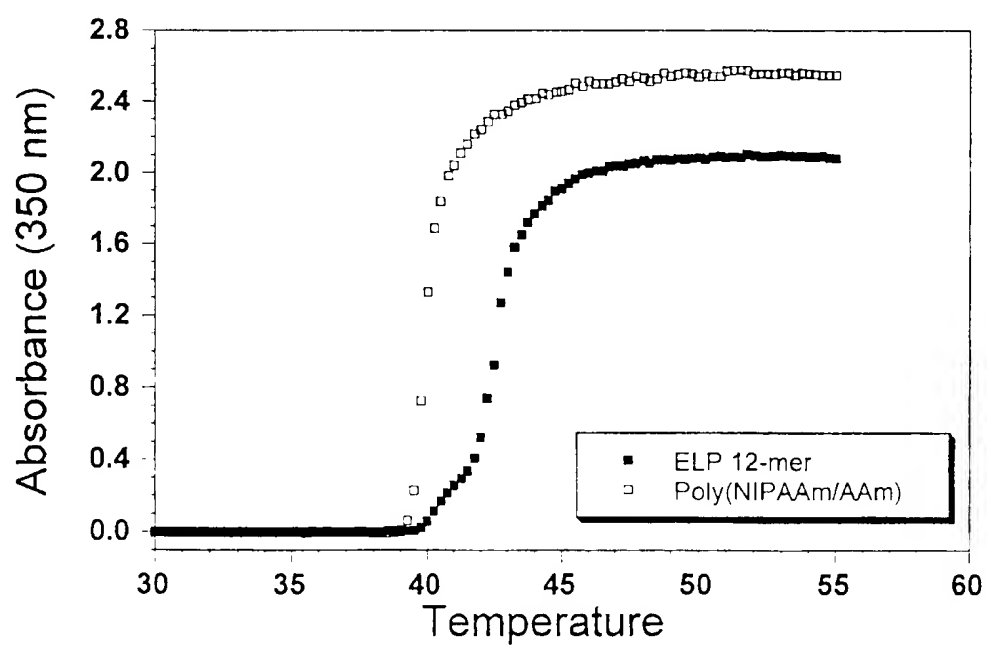


Figure 9

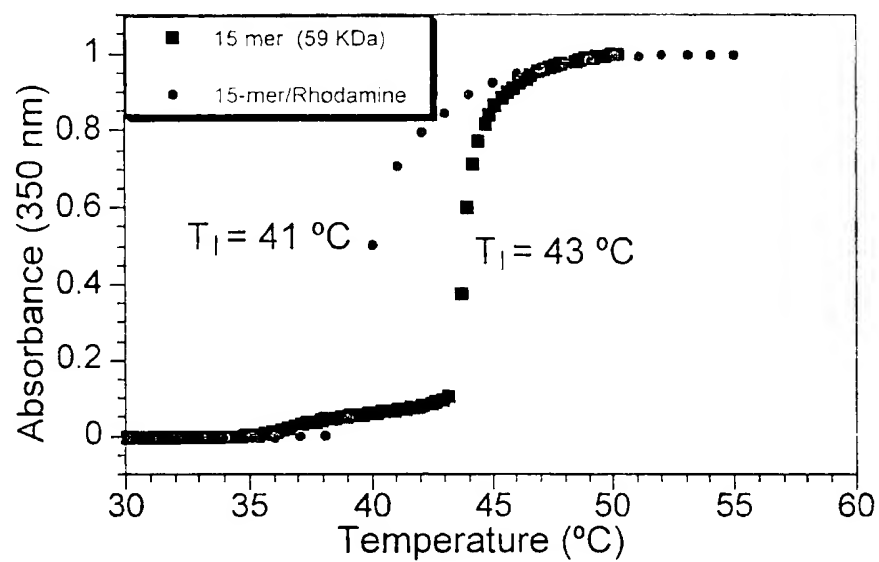
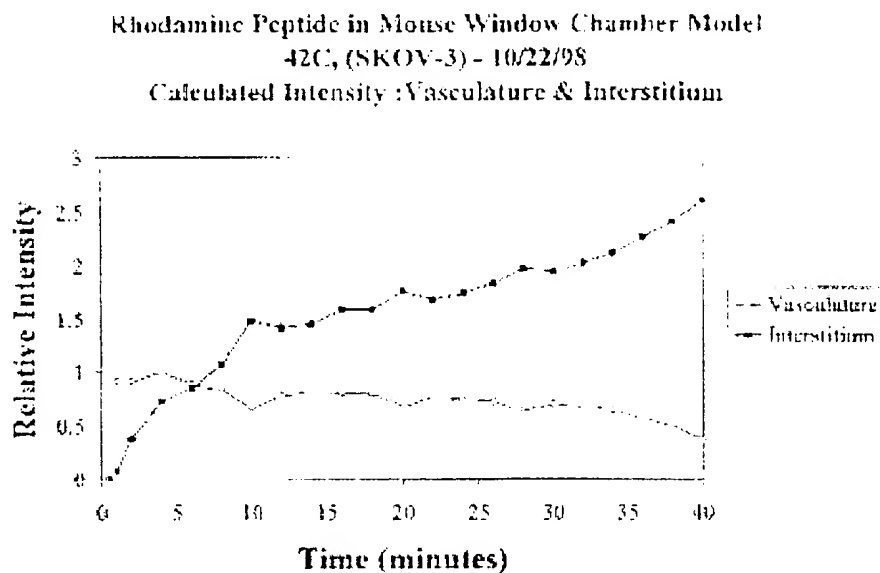


Figure 10

A.



B

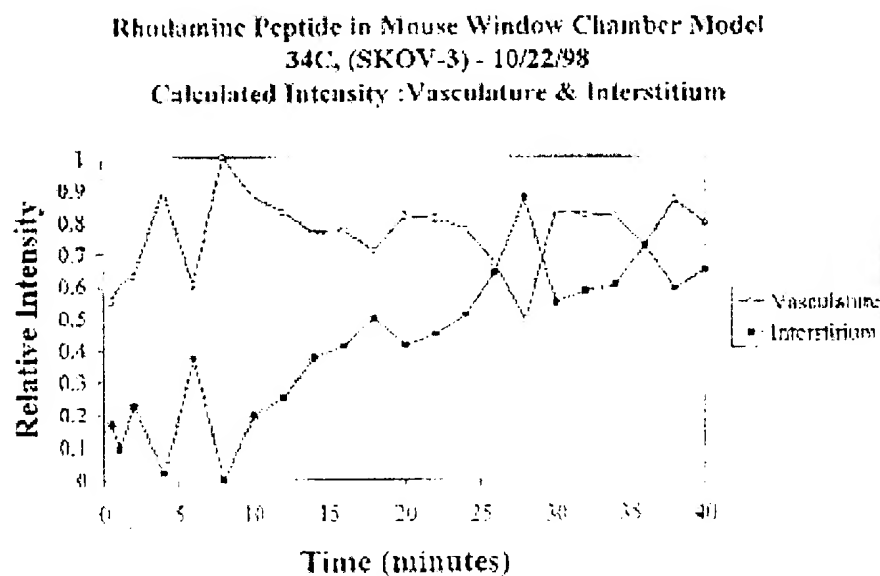
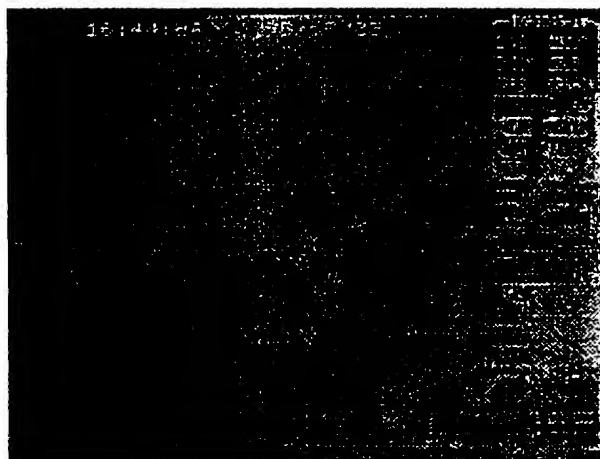


Figure 11

(A)



(B)



Figure 12

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/05780

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) : C07K 17/08; A61K 39/385, 39/395 US CL : Please See Extra Sheet. According to International Patent Classification (IPC) or to both national classification and IPC														
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 530/402, 350, 387.1, 391.1, 395, 399, 403, 404, 405, 406; 424/78.08, 178.1, 193.1, 280.1, 94.1 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN, BIOSIS, MEDLINE, USPATFUL, CAPLUS														
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>														
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
Y	US 4,474,851 A (URRY) 02 October 1984, see entire document.	11-20												
Y	US 5,816,259 A (ROSE) 06 October 1998, see entire document.	31-40												
Y,P	US 5,900,405 A (URRY) 04 May 1999, see entire document.	11-27												
Y,P	US 5,998,588 A (HOFFMAN et al) 07 December 1999, see entire document.	1-27, 31-40												
Y	CHEN et al. Polymer-protein conjugates. Biomaterials. 1990, Vol. 11, pages 631-634, see entire document.	1-10												
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents</td> <td>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>*A* document defining the general state of the art which is not considered to be of particular relevance</td> <td>*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>*B* earlier document published on or after the international filing date</td> <td>*Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date or another citation or other special reason (as specified)</td> <td>*A* document member of the same patent family</td> </tr> <tr> <td>*C* document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>*P* document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	*B* earlier document published on or after the international filing date	*Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date or another citation or other special reason (as specified)	*A* document member of the same patent family	*C* document referring to an oral disclosure, use, exhibition or other means		*P* document published prior to the international filing date but later than the priority date claimed	
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Date of the actual completion of the international search 22 MAY 2000		Date of mailing of the international search report 15 AUG 2000												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks P.O. Box 108 Washington, DC 20540		Authorized officer JOYCE BRIDGERS PARALEGAL SPECIALIST M.A. 10/10/00												

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/05780

## C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEN et al. Preparation and Properties of Thermoreversible, Phase-Separating Enzyme-Oligo(N-isopropylacrylamide) Conjugates. Bioconjugate Chemistry. 1993, Vol. 4, pages 509-514, see entire document.	1-20
Y	GALAEV et al. Affinity Thermoprecipitation: Contribution of the Efficiency of ligand-Protein Interaction and Access of the Ligand. Biotechnology & Bioengineering. 1993, Vol. 41, pages 1101-1106, see entire document.	1-20
Y	CHEN et al. A new temperature- and pH-responsive copolymer for possible use in protein conjugation. Macromol. Chem. Phys. 1995, Vol. 196, pages 1251-1259, see entire document.	1-20
Y	MAEDA et al. Modification of DNA with poly(N-isopropylacrylamide) for thermally induced affinity separation. Reactive Polymers. 1993, Vol. 21, pages 27-35, see entire document.	1-20
Y	MONJI et al. Application of a thermally-reversible polymer-antibody conjugate in a novel membrane-based immunoassay. Biochemical & Biophysical Research Communications. 1990, Vol. 172, No. 2, pages 652-660, see entire document.	1-27, 31-40
Y	MONJI et al. A novel immunoassay system and bioseparation process based on thermal phase separating polymers. Applied Biochemistry & Biotechnology. 1987, Vol. 14, pages 107-120, see entire document.	1-20
Y	STAYTON et al. Control of protein-ligand recognition using a stimuli-responsive polymer. Nature. 1995, Vol. 378, pages 472-474, see entire document.	1-20
Y	TAKEI et al. Temperature-responsive bioconjugates. 2. Molecular design for temperature-modulated bioseparations. Bioconjugate Chem. 1993, Vol. 4, pages 341-346, see entire document.	1-20
Y	TAKEI et al. Temperature-responsive bioconjugates. 1. Synthesis of temperature-responsive oligomers with reactive end groups and their coupling to biomolecules. Bioconjugate Chem. 1993, Vol. 4, pages 42-46, see entire document.	1-20
Y	TAKEI et al. Temperature-responsive bioconjugates. 3. Antibody-Poly(N-isopropylacrylamide) conjugates for temperature-modulated precipitations and affinity bioseparations. Bioconjugate chem 1994, Vol. 5, pages 577-582, see entire document.	1-20



# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US00/05780

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	UMENO et al. Single stranded DNA-poly(N-isopropylacrylamide)conjugate for affinity precipitation separation of oligonucleotides. Chem. Commun. 1998, pages 1433-1434.	1-27, 31-40
A,P	UMENO et al. Temperature-induced precipitation of specific DNA fragments using DNA-poly(N-isopropylacrylamide) conjugate. Chemistry Letters. 1999, pages 381-382.	1-27, 31-40
Y	NGUYEN et al. Syntheses and applications of water soluble reactive polymers for purification and immobilization of biomolecules. Biotechnology & Bioengineering. 1989, Vol. 34, pages 1186-1190, see entire document.	1-27, 31-40
Y	WU et al. Conjugation of phosphatidylethanolamide to poly(N-isopropylacrylamide) for potential use in liposoma drug delivery systems. Polymer. 1992. Vol. 33, No. 21, pages 4659-4662, see entire document.	31-40

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/05780

## A. CLASSIFICATION OF SUBJECT MATTER

US CL :

530/402, 350, 387.1, 391.1, 395, 399, 403, 404, 405, 406; 424/78.08, 178.1, 193.1, 280.1, 94.1

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-10, drawn to an immunoassay method.

Group II, claim(s) 11-20, drawn to an article useful as a regenerable biosensor.

Group III, claim(s) 21-27, drawn to a method of recycling a biosensor.

Group IV, claims 31-40, drawn to a method for in vivo delivering of a target compound.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the instant invention is the bioelastomer conjugate comprising the bioelastomer and a protein or peptide. Such a compound is not novel and thus is not considered to be inventive.

Chen et al (Biomaterials, Vol. 11, pages 631-634, 1990) disclose a polymer-protein conjugates comprising protein A and poly(N-isopropylacrylamide) for use in affinity precipitation-based immunoassay where the conjugate is incubated with an unknown mixture and heated to precipitate quantitatively the antigenic analyte.